

PATHOGENETIC MECHANISMS AND HOST RESPONSES IN GIARDIASIS -
STUDIES IN PATIENTS AND IN LABORATORY ANIMALS

THESIS PRESENTED TO THE UNIVERSITY OF EDINBURGH
FOR THE DEGREE OF M.D.
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MAY 1983



FOR MY PARENTS

ACKNOWLEDGMENTS

This thesis would not have been possible without the help, encouragement and expert knowledge of Dr. Anne Ferguson, to whom I am sincerely grateful.

I am also indebted to Dr. Claude André, who welcomed me into his laboratory and whose theoretical knowledge, technical expertise and enthusiasm facilitated the immunological studies described in Chapters 5 and 9.

The clinical studies of giardiasis were undertaken with the help of the medical and nursing staff of the Gastrointestinal Unit, Western General Hospital, Edinburgh, and in particular I would like to thank Dr. W. Sircus for allowing me to study patients under his care.

ACKNOWLEDGMENT OF TECHNICAL AND COLLABORATIVE ASSISTANCE

From the onset of the work described in this thesis I was involved in the conception and execution of the studies, making myself familiar with the techniques involved. In many instances, however, I was heavily dependent on others for their expert technical assistance, and wish to express my thanks to all of those mentioned below.

Miss Gillian Paul, Mrs Frances Hay and Mr. A Sutherland performed most of the technical work reported in Chapter 7, i.e. morphological measurements and cell kinetic studies in the mouse small intestine. They were also responsible for the maintenance of the animal model of giardiasis in the laboratory, and I am grateful to them for their help in some of the work which I myself performed, namely the development of the vibration method for trophozoite counts and the studies of intraepithelial lymphocytes.

Dr. D. Al-Thamery was responsible for the disaccharidase estimations reported in Chapter 7, while Dr. K.V. Nair helped in the experiments on the effects of corticosteroids described in Chapter 6.

The immunofluorescence studies described in Chapters 5 and 9 were performed in Dr. Claude André's laboratory at the Hôpital Edouard Herriot, Lyon. In both instances considerable technical assistance was afforded by M. Gérard Jourdan and Miss Marie-Claire Fargier.

Dr. I. Roberts-Thomson (Melbourne, Australia), who provided the initial cyst inoculum allowing the animal

model to be established in Edinburgh, kindly agreed to collaborate in our studies of intraepithelial lymphocytes by supplying histological specimens from nude mice infected with Giardia muris, according to an agreed protocol.

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REFERENCES

The following papers concerning work described in this thesis have been published.

Nair KV, Gillon J, Ferguson A. Corticosteroid treatment increases parasite numbers in murine giardiasis. Gut 1981; 22 : 475 - 480.

Gillon J, André C, Descos L, Minaire Y, Fargier MC. Changes in mucosal class-specific immunoglobulin-containing cells in patients with giardiasis before and after treatment. J. Infect. 1982; 5 : 67 - 72.

Gillon J, Al-Thamery D, Ferguson A. Features of small intestinal pathology (epithelial cell kinetics, intraepithelial lymphocytes, disaccharidases) in a primary Giardia muris infection. Gut 1982; 23 : 498 - 506.

The data in Chapter 3 were presented to the Caledonian Society of Gastroenterology in June 1981 under the title 'Giardiasis in an adult Gastro-intestinal Unit'.

ADDENDA

Throughout this thesis the terms "giardiasis" and "infection with Giardia" are synonymous.

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The term "cytolysosome" is quoted from the paper to which reference is made, and is taken to refer to intracytoplasmic granules with features typical of lysosomes.

Abbreviations

| | | |
|---------|---------|--------------------------|
| Page 46 | HCl | - hydrochloric acid |
| | H and E | - haematoxylin and eosin |

ABSTRACT

The studies described in this thesis examine two aspects of this common enteric infection, namely the pathogenesis of the intestinal damage and malfunction and the host immune response, and the relationship between immunological mechanisms and pathological changes. My own experience of the disease in adults is described, detailing the functional and pathological consequences of giardiasis. Immunological studies in humans consisted of, firstly, an immunofluorescence study of immunoglobulin-containing cells in the jejunum. This showed a striking increase in IgE-cells, with prompt reversal after treatment, and a slight decrease in IgA cells with subsequent significant increase. Secondly, counts of intra-epithelial lymphocytes (IEL) were performed on jejunal biopsies before and after treatment. Raised counts were only found in those with villous atrophy, and reversed after treatment.

The animal model was used to extend these studies. New methods were developed for accurate measurement of trophozoite numbers in the small intestine of infected mice, and these, and the new information they provided on the course of infection and distribution of parasites, are described in detail. In up to 50% of CBA mice trophozoites could be detected in the intestine many months after apparent self-cure, a state of latent infection hitherto unsuspected. The pathological effects of the organism on small intestinal morphology, epithelial cell kinetics and brush border enzymes

were studied sequentially throughout the course of a primary infection. The results indicate that the parasite has a direct damaging effect on the jejunal epithelium at the time of the infection, with reduction of villus height and disaccharidases. A 'rebound' increase occurred after self-cure. Pathological changes in the ileum were the reverse of those seen in the jejunum, demonstrating the rapidity of ileal adaptation in the presence of jejunal dysfunction.

The animal model was also used for immunological studies. Sequential IEL counts in normal and athymic mice showed that IEL numbers rose at the time of self-cure, and remained high for many weeks. This did not occur in the athymic mice unless reconstituted with viable lymphoid cells. The effect of corticosteroids on primary and latent infection was assessed. Primary infection was heavier in corticosteroid-treated animals and recrudescence of latent infection occurred. Finally the animal model was used to develop an immunofluorescent antibody assay, and the kinetics of the antibody response following primary and secondary infection were studied. In BALB/c mice peak antibody titre (IgG) did not occur until after self-cure, and no 'booster' response occurred after subsequent oral inoculation with *G. muris*. These results suggest that systemic antibodies play little part in elimination of *Giardia* by a normal host.

CHAPTER 1

INTRODUCTION AND AIMS OF THESIS

The flagellate protozoon Giardia lamblia was first described in 1681 by van Leevenhoeck, who identified the parasite in his own faeces while experimenting with his newly invented microscope. Historically the organism was regarded as a harmless commensal until the past two decades. Although it may indeed cause an infection which remains asymptomatic, it is now recognised as an important pathogen throughout the world and particularly in regions where sanitation is poor. Malabsorption of various substances has been well documented in subjects with giardiasis, but the histological abnormalities in the small intestinal mucosa have been described somewhat sketchily and the pathogenesis of the malabsorption is poorly understood. The immune response to the parasite has received much attention, but with little attempt to relate the changes observed to other aspects of the disease.

I was first attracted to the study of this condition when, on coming to work in the Gastro-intestinal Unit with Dr. Anne Ferguson, I learned of the animal model of the infection which she had established in her laboratory. Using this model and my own clinical experience of the disease in parallel I have addressed myself to several aspects of the host-parasite relationship.

During the period in which these studies were carried out I was able to perform extensive investigations on all patients with giardiasis presenting to the Gastro-intestinal Unit. This entailed detailed study of

clinical features, small intestinal histology and indices of malabsorption. In Lyon, France, where I spent a year in the laboratory of Dr. Claude André, I was able to extend this work by enumerating the immunoglobulin producing cells of the small intestine in patients with giardiasis, and in both centres measurements of intraepithelial lymphocytes were performed.

Such studies were designed to confirm and extend previous observations on the intestinal changes in giardiasis, to document the effects of treatment and identify any predisposing factors in these individuals.

The amount of information that can be derived from such human work is clearly limited both by the variation in individual response to the parasite and, more particularly, by the need for invasive investigations such as jejunal biopsy. Giardiasis is, however, a naturally occurring infection in many other species, including the mouse, and the murine model of the infection offers many advantages, the most important of which are as follows:

1. Although different genetic strains of mice respond differently to the parasite, this can be held constant in any given experiment, with a uniform and reproducible result.
2. The infecting dose of the organism can be controlled and parasite numbers documented.
3. The whole intestine of the animal is available for study.

4. Observations may be made at regular intervals throughout the course of the infection.
5. Parasite numbers may be manipulated, for instance by appropriate antimicrobial therapy, and the host response modified by various means, e.g. altering nutritional status or treating with immunosuppressive drugs.

Given that such detailed observations are possible, and since the relevance of such data to the human disease is probable, the disease occurring naturally in mice, experiments were designed to examine the intimate relationship between the parasite and the intestinal mucosa. To this end it seemed of paramount importance to be able to ascertain the exact magnitude of the parasite load at any given time in the course of the infection. I therefore developed the method of counting trophozoites described in Chapter 5, and in addition to this a different method was devised for examining the distribution of the parasite in the small intestine. Using these new methods we quickly discovered that after the animals had cured themselves of the disease, as most strains do within a few weeks, small numbers of parasites may remain viable in the small intestine for many months. This phenomenon was examined further and the effects of immuno-suppression with corticosteroids on both this latent infection and the primary acute infection were studied.

The information gained using these techniques was correlated with measurements of intestinal architecture,

cell turnover and disaccharidases throughout the course of the infection. Time-course studies of intraepithelial lymphocytes as a measure of mucosal cell-mediated immunity were also carried out. Thus, at intervals throughout the duration of the infection I sought to compare the pathological changes observed with exact parasite numbers at different levels in the small intestine, in the hope of being able to establish which of the abnormalities may be due to the direct action of the parasite itself, and which may have some other basis.

Finally, the murine model was used to study the systemic antibody response to the parasite. Serological studies in humans have proved difficult to develop, and their interpretation may be facilitated by an understanding of the antibody response in mice during, and following, a controlled infection. If there is to be any prospect of the development of an effective vaccine to giardiasis, such information may be of considerable value.

CHAPTER 2

GIARDIASIS - A REVIEW OF THE LITERATURE

INTRODUCTION

Giardia organisms are widely distributed intestinal parasites of all classes of vertebrates. Unfortunately considerable confusion has been caused by the lack of agreement on nomenclature. Two genus names are currently used, Giardia being common in the English speaking world, and Lamblia, after the Czeck, Lambl, who 'rediscovered' the organism in the diarrhoeal stool of a child in 1859, in the rest of Europe and the Soviet Union. On the basis of such criteria as host specificity and morphological features many different species of Giardia have been proposed, but there are now many reports which indicate that host specificity is far from rigid. The complexities of Giardia nomenclature and biology are well reviewed by Meyer and Radulescu (1979), who conclude that the classification of Filice (1952) should be accepted until more concrete evidence of species differences is obtained. He proposed three species of Giardia:

Giardia agilis, affecting mainly tadpoles and frogs.

Giardia muris, a form found mainly in birds
and rodents.

Giardia duodenalis, the organism which affects many
mammalian species, including man,
dog and rabbit.

While accepting that this approach is sensible, I have preferred in this thesis to use the term Giardia lamblia rather than Giardia duodenalis when discussing

human infection, as this is still the terminology used in most medical literature on the subject. The term Giardia muris is used in the sections dealing with experimental infection in animals.

Giardia organisms exist in two forms. The trophozoite is a mobile flagellate which is found in the small intestine, where it obtains nourishment by diffusion through the cell wall. It is closely adherent to the intestinal mucosa, where it may rapidly establish its presence in great numbers, dividing by binary fission every five hours or so (de Carneri et al., 1977). The cystic form is thought to develop in the lower ileum or colon, and is excreted in the faeces. The cysts are rapidly destroyed by drying, but may survive in wet conditions and remain infective for 2 months or more (Bingham et al., 1979). Excystation occurs in the stomach or upper small intestine of the host, and it has been shown that in man infection may be caused by as few as 10 cysts (Rendtorff 1954). Many studies have now been published establishing beyond doubt that Giardia, for long regarded as a harmless commensal, is a common intestinal pathogen in humans (reviewed by Meyer and Radulescu, 1979).

GIARDIASIS IN TEMPERATE ZONES

The epidemiology of giardiasis has been well reviewed in recent years (Wolfe 1978, Amin 1979, Meyer and

Jarroll 1980, Smith and Wolfe 1980). Although commonest in areas such as the tropics, where sanitation is poor (Smith and Wolfe 1980) the disease is also seen sporadically in non-tropical areas (Petersen 1972, Meuwissen et al., 1977), and it is now regarded as endemic in the USA (Smith and Wolfe 1980). Infection is probably transmitted mainly by the faecal-oral route (Meyer and Jarroll 1980), but epidemics have occurred when parties of susceptible individuals have visited areas where water supplies were contaminated with Giardia cysts (Moore et al., 1969, Fiumara 1973, Brodsky et al., 1974, Shaw et al., 1977). Direct person-to-person spread is also known to occur, notably in closed communities such as children's institutions (Black et al., 1977) and among male homosexuals (Meyers et al., 1977, Schmerin et al., 1978).

In a review of 24 published surveys of nearly 36,000 subjects, Quinn (1971) arrived at a prevalence in the United States of 7.4%. In a hospitalised group of Norwegian patients the incidence was 3.2% using stool examination as the method of detection (Petersen 1972). When 293 patients with gastro-intestinal symptoms were subjected to duodenal intubation Giardia trophozoites were detected in 6.7%, and when patients with diarrhoea were considered separately the incidence rose to 15% (Petersen 1972). Faecal cyst detection is known to be an unreliable method for the diagnosis of giardiasis, since many subjects excrete cysts only intermittently even at the height of the infection (Ament and Rubin 1972, Kamath and Murugasu 1974), and more

accurate estimation of the prevalence of the disorder will only become possible when reliable serological tests become widely available. Efforts in this direction have hitherto been thwarted by the lack of a consistent source of antigenic material, but culture of the organism in the laboratory has recently been shown to be possible (Meyer 1976, Gillin and Diamond 1980). Preliminary serological studies in humans, using these cultured trophozoites, indicate that the incidence of previous infection may be as high as 14% in the USA (Smith et al., 1981). In 1977 Giardia was reported to be the most frequently detected bowel pathogen in public health laboratories in the United Kingdom (Communicable Disease Weekly Reports, 1977), and since this depends on faecal cyst detection, which is inaccurate even when concentration methods are used, it gives an indication of how common the infection may be in the community. To date no attempt has been made to assess the prevalence of the disease in this country using existing methods, nor to estimate the magnitude of the problem of giardiasis in hospital practice.

CLINICAL FEATURES OF GIARDIASIS IN HUMANS

In a series of experiments in which he infected human volunteers Rendtorff (1954) demonstrated that the infection may be asymptomatic or may cause a short-lived

diarrhoeal illness. Most subjects eradicated the parasite spontaneously. It is now known that some asymptomatic carriers may excrete the cysts for many years (Danciger and Lopez, 1975). There is no doubt that the parasite is a common cause of diarrhoeal illness both in the tropics (Antia et al., 1966), and in temperate zones (Meuwissen et al., 1977). It can give rise to an acute, self-limiting diarrhoea with or without abdominal discomfort and vague upper gastro-intestinal symptoms, to a chronic, intermittent diarrhoea with distention and flatulence, or to a malabsorption syndrome (Antia et al., 1966; Ament and Rubin 1972; Wright et al., 1977; Yardley and Bayless, 1967). Infected patients may remain symptomatic for years (Alp and Hislop 1969).

The reasons for the extreme individual variation in the response to the parasite remain poorly understood. Rendtorff (1954) showed that the size of the infecting dose was an important variable, but it is likely that host-related factors are most important in determining the outcome of infection (Yardley and Bayless, 1967)*. Children are probably more vulnerable than adults (Veghelyi 1938, Meuwissen et al., 1977), as are malnourished individuals (Yardley and Bayless, 1967) and those with impaired immune functions, particularly hypogammaglobulinaemia (Ament and Rubin, 1972). It has been suggested that gastrectomy might also be a predisposing factor in adults, the presumed reason being the decrease in gastric acidity (Vachon et al., 1963; Yardley et al., 1964; Gianella et al., 1973). These

* Subjects with the blood group A phenotype appear to have an increased risk of symptomatic giardiasis (Barnes and Kay, 1977; Zisman, 1977).

groups of patients have an increased risk of developing a malabsorption syndrome in association with giardiasis (Meyer and Radulescu 1979, José and Welch 1970, Kamien et al., 1974), but there are many reports indicating that giardiasis can, of itself, cause malabsorption (Zamchek et al., 1963; Yardley et al., 1964; Antia et al., 1966; Morecki and Parker 1967; Hoskins et al., 1967; Cain et al., 1968; Alp and Hislop, 1969; Wright et al., 1977), not only of fat but also of vitamin A (Ember and Mindszenty 1969), and vitamin B₁₂ (Antia et al., 1966, Ament and Rubin 1972, Wright et al., 1977).

PATHOGENESIS OF MALABSORPTION

Malabsorption of fat

The mechanism whereby giardiasis causes malabsorption has long been disputed. Veghelyi (1940) believed that the number of parasites could become so great that a mechanical barrier to absorption was formed. Chawla et al. (1975), and Gupta and Mehta (1973) suggested that secondary pancreatic insufficiency may be the cause of malabsorption, while Tandon et al., (1977) reported their attempts to prove that bacterial overgrowth might accompany giardiasis and lead to deconjugation of bile salts within the intestinal lumen. They found that almost 50% of their patients with giardiasis and malabsorption had significant bacterial overgrowth in the duodenum, but on the other

hand several of those with no evidence of bacterial overgrowth, and two patients with giardiasis but no evidence of malabsorption, had abnormally large quantities of free bile salts in the duodenal aspirate. Bile salts appear to be necessary for the survival of Giardia trophozoites (Bemrick 1963), and it is therefore possible that the parasites themselves contribute to the malabsorption process by affecting bile salt metabolism.

The question of whether malabsorption may be mainly due to parasite-related damage to the intestinal mucosa has not been answered satisfactorily. Both in patients with immunodeficiency syndromes (Ament and Rubin 1972), and in immunologically intact individuals (Hoskins et al., 1967, Wright et al., 1977, Duncombe et al., 1978), clinically obvious malabsorption appeared to be associated with severe histological abnormality, and both Ament and Rubin and Wright et al. showed that there was a rough correlation between the degree of malabsorption and the amount of mucosal damage. In these studies, however, the authors had to rely entirely on biopsies from the upper jejunum, and no information on the magnitude of the parasite burden or its distribution in the small intestine could be obtained. Changes in indices of malabsorption, histological abnormalities and parasite numbers with time have not been studied adequately in humans, largely because of the difficulty in repeating invasive investigations. From the above studies, however, it can be said that fat malabsorption is likely to

be due mainly to the effects of the parasite on the small intestinal mucosa.

Malabsorption of Vitamin B₁₂

It is not clear whether the parasite utilises vitamin B₁₂ within the lumen. Scudamore et al., (1961) could demonstrate no abnormality of B₁₂ absorption in patients with giardiasis, but Ament and Rubin (1972) found abnormal Schilling tests in six of eight immunodeficient patients with giardiasis. However, eradication of the parasite led to improved B₁₂ absorption in only half of these patients, who are, in any case, known to be predisposed to B₁₂ malabsorption caused by intrinsic factor deficiency. Wright et al., (1977) found evidence of B₁₂ malabsorption in 20 of their 40 patients with giardiasis, none of whom was immunodeficient. The degree of B₁₂ malabsorption seemed to correlate with the severity of histological change in the intestine, and absorption returned to normal in six of nine patients treated with metronidazole, three of four treated with mepacrine and two of four treated with tetracycline. The effect of tetracycline perhaps indicates that associated bacterial colonisation is important, but on balance the data suggest that any deficiency in B₁₂ absorption is related more to mucosal damage than to utilisation of the vitamin by the parasite.

PATHOLOGICAL CHANGES IN THE MUCOSA OF THE
SMALL INTESTINE IN HUMAN GIARDIASIS

Histological changes in patients without immunodeficiency

The earlier published reports of the pathological changes in the small intestine in patients with giardiasis were often based on small numbers of patients, but were valuable in helping to establish that the infection could cause a clinical syndrome accompanied by pathological damage to the mucosa. Though anecdotal and occasionally lacking in detail these papers were important in attracting attention to giardiasis, those which included the effects of specific anti-Giardia treatment being especially necessary to dispel scepticism.

Zamchek et al., (1963) described three patients with diarrhoea due to giardiasis in whom the jejunal morphology ranged from normal to sub-total villus atrophy. Both acute (polymorphonuclear leucocytes and eosinophils) and chronic inflammatory cells were noted in the lamina propria, and the number of epithelial mitoses was thought to be greatly increased. These changes reverted to normal after treatment. Yardley et al., (1964) documented "moderate" villus atrophy in three of their six patients, and again recorded the presence of both acute and chronic inflammatory cells in the lamina propria. In addition to this they found eosinophils and polymorphs within the epithelial layer, and at these sites the epithelial cells showed evidence of damage. They also noted increased mitotic figures in crypts

in five of their six patients. The result of treatment was not recorded. Morecki and Parker (1967) and Brandborg et al., (1967) reported that patients with symptomatic giardiasis could have normal jejunal histology with no inflammatory infiltrate, though Brandborg et al. occasionally observed necrotic epithelial cells and loss of nuclear polarity. In a careful and detailed study, again using a small number of patients, Hoskins et al. (1967) reported a spectrum of morphological change from normal to almost complete villus atrophy. The only epithelial abnormality was loss of polarity of the nuclei, but acute and chronic inflammatory cells were again seen in the crypt epithelium as well as in the lamina propria. They noted that crypt mitoses appeared to be increased in proportion to the degree of villus damage. The most important aspect of their study was the attention they gave to the effect of treatment. In one patient with severe mucosal damage the changes had returned almost to normal when re-biopsied only two days after completing treatment with Atabrine. In a second patient re-biopsied at ten days a similar response occurred. The significance of this very prompt response of the mucosa to eradication of the parasite has largely been lost on later investigators.

Barbieri et al., (1970), using jejunal biopsies from children with giardiasis, found only very minimal villus damage but recorded the presence of both acute and chronic inflammatory cells in the lamina propria. More recent studies (Wright et al., 1977, Duncombe et al., 1978) have

confirmed a range of histological change from normal to subtotal villus atrophy. Duncombe et al., (1978) found that the density of the inflammatory infiltrate in the lamina propria was greater when villus atrophy was present. Both groups reported almost complete reversal of histological changes following successful treatment, but in neither study was the time course of the response to treatment documented.

Histological changes in hypogammaglobulinaemic patients with giardiasis

Studies of patients presenting de novo with symptomatic giardiasis have usually revealed no underlying abnormality of serum immunoglobulins (Jones and Brown, 1974, Wright et al., 1977, Geller et al., 1978). Nevertheless, a high incidence of giardiasis has been shown to occur in patients with immunodeficiency syndromes, especially common variable hypogammaglobulinaemia (Ament et al., 1973). It was the commonest cause of diarrhoea in these patients, and was associated with abnormalities of mucosal architecture ranging from mild to severe villus atrophy. No plasma cells were visible in the lamina propria, but no comment was made on the presence of other inflammatory cells. In a previous study (Ament and Rubin, 1972) two of seven patients with giardiasis had nodular lymphoid hyperplasia, two had severe villus atrophy and the remaining three had mixed lesions. The severity of villus damage seemed to correlate with the degree of malabsorption. In both studies successful

treatment of giardiasis led to symptomatic cure and improvement in all abnormalities of mucosal architecture except nodular lymphoid hyperplasia.

Although no direct comparisons have been reported, it seems likely from the above papers that giardiasis produces a more severe degree of villus damage in hypogammaglobulinaemic patients than in otherwise normal individuals.

Ultrastructural abnormalities of mucosal morphology

Electron microscopic studies in man have usually revealed the epithelial cells to be normal (Morecki and Parker, 1967, Hoskins et al., 1967) or showing only mild abnormalities, for example local cytoplasmic alterations in cells adjacent to inflammatory cells (Takano and Yardley 1965) or inclusion bodies thought to be "cytolysosomes" (Barbieri et al., 1970). The most consistent abnormality reported is shortening and distortion of the microvillus border (Takano and Yardley, 1965; Barbieri et al., 1970; Hoskins et al., 1967). Barbieri et al., (1970) also found thickening of the "fuzzy coat". These abnormalities have also been described in rats with bacterial colonisation of the small bowel (Wehman et al., 1978).

Abnormalities of jejunal disaccharidases in giardiasis patients

Hoskins et al., (1967) reported a patient with lowered jejunal disaccharidases (lactase, sucrase and maltase) and a flat oral lactose tolerance test, with only mild

abnormalities of mucosal architecture. These changes reverted to normal within ten days of successful treatment of his giardiasis. Similar findings were reported by Cain et al., (1968), the patient having normal villus architecture. However the patient continued to have milk induced symptoms, a flat lactose tolerance test and slightly reduced lactase level (which had been unrecordable at the time of infection), when studied one year later. These authors wondered whether pre-existing lactase deficiency might predispose to giardiasis. Campbell et al., (1972) reported two patients in whom all three disaccharidases tested were reduced at the time of infection. No histological details were given. One patient was studied at an undisclosed interval after treatment with metronidazole and all values had returned to normal. In the same year Ament and Rubin (1972) found abnormal lactose tolerance tests in three patients with giardiasis out of six immunodeficient patients studied. One of these patients, however, had normal disaccharidase values, as did one patient with nodular lymphoid hyperplasia who also had normal lactose tolerance. The two patients who had both abnormal disaccharidases and reduced lactose tolerance both had mixed villus atrophy and lymphoid nodules, and in both the enzyme abnormalities returned to normal after eradication of giardiasis (time interval undisclosed).

Studies involving larger numbers of patients have been reported by Jennings et al., (1976) and Duncombe et al., (1978). The former reported that all of their 11 patients

with giardiasis had reduced disaccharidases, and three of these had symptomatic milk intolerance which recovered after treatment. Five patients had normal histology, four had mild abnormalities and two had moderately severe villus atrophy. The three symptomatic patients had only mild histological changes. Disaccharidase estimations were not repeated after treatment.

Duncombe et al., (1978) studied disaccharidase levels in 16 patients and found that all patients with significant diarrhoea had reduced lactase. Very low lactase levels occurred in patients with histology graded as normal or only mildly abnormal as well as in those with subtotal villus atrophy. Measurements were repeated in three patients after treatment. In two the disaccharidases had returned to normal, but lactase remained low in the third, whose symptoms and histology also failed to show improvement.

It is clear that disaccharidase deficiency is common in giardiasis, and can occur when only minimal abnormalities are evident on light microscopy. Frank lactose intolerance appears, however, to be relatively unusual.

The studies referred to above established beyond doubt that Giardia organisms are pathogenic to the human gastro-intestinal tract. Many questions remained unanswered; for example little was known of the pathogenetic mechanisms or of the host's response to the parasite, and the self-cure phenomenon and subsequent resistance to reinfection described by Rendtorff (1954) also required detailed studies for their elucidation. Such studies are difficult to perform in humans, and the animal model of the disease first described by Roberts-Thomson et al. (1976(a)) has therefore proved invaluable. This animal model provides the basis for all of the experimental work on mice described in this thesis.

EXPERIMENTAL GIARDIASIS IN MICE

Giardiasis occurs in many vertebrates other than man, including laboratory rodents. The experimental model of murine giardiasis first described by Roberts-Thomson et al. (1976) has proved useful for studies of host-parasite relationships, since changes in the host intestine can be studied in sequence and with precise knowledge of parasite numbers and distribution. The animals used in such experiments do not become ill, and in the strains of mice first examined by Roberts-Thomson et al., the infection, as judged by faecal cyst counts and a crude estimation of trophozoite numbers in the small intestine, reached a peak of severity around the second to third weeks after inoculation, and most animals appeared to have effected self-cure by weeks 6 to 8.

Pathological changes in the small intestine in murine giardiasis

Although the animal model of giardiasis has for the most part been used by Roberts-Thomson and his colleagues, and by other workers, to study the host immune response into Giardia infection (described below), a limited amount of information on the pathological changes engendered by the parasite in the small intestine has been obtained. In their original description Roberts-Thomson et al., (1976) reported that in CF-1 mice the villus to crypt ratio was significantly reduced in the upper jejunum at the time of maximum trophozoite numbers, regardless of the size of the cyst

inoculation. In animals given a large inoculation (10,000 cysts) the ratio fell by day 3 and had recovered by day 14, whereas in animals given 100 or 1000 cysts the changes developed more slowly and took longer to recover. No abnormality of mucosal architecture was found in the mid small bowel or distal ileum.

McDonald and Ferguson (1978), studying different strains of infected mice, including one group of animals in which the disease had become endemic, reported that although the histology of the small intestine appeared normal, the infected mice had significantly longer crypts than uninfected controls. No differences in villus height were detected between the groups, but dynamic studies of crypt cell production rate showed that the rate of cell turnover doubled in chronically infected animals. Tissue disaccharidase levels were studied in some of these groups of mice, but no consistent pattern of abnormality was observed.

THE IMMUNE RESPONSE IN GIARDIASIS

In the series of experiments referred to above in which he infected human volunteers with cysts of *Giardia lamblia*, Rendtorff (1954) made the observation that there was a tendency for infected subjects to become resistant to infection on subsequent exposure to the organism. He made no comment on the significance of this finding.

Although many authors subsequently described a marked inflammatory response in the intestinal mucosa (Zamchek et al., 1963, Yardley et al., 1964, Hoskins et al., 1967, Barbieri et al., 1970), the importance of the host immune response in giardiasis only became clear when this was shown to be a very frequently occurring disorder in patients with hypogammaglobulinaemia (Ament and Rubin, 1972). The intestinal immune response to Giardia is of particular interest since the organism does not normally penetrate the mucosa.

HOST IMMUNITY IN HUMAN GIARDIASIS

That an immune response occurs in infected individuals is suggested by the observed resistance to subsequent infection (Rendtorff 1954), the association of giardiasis with various allergic phenomena (described below), and the increased susceptibility of patients with hypogammaglobulinaemia (Ament and Rubin 1972).

The allergic manifestations reported to occur in association with giardiasis include bronchial asthma (Fossati 1971, Lopez-Brea et al., 1979), urticaria (Harris and Mitchell 1949, Dellamonica et al., 1976, Weisman 1979), arthritis (Goobar 1977), uveitis (Carroll et al., 1961) and food intolerance (Halstead and Sadun 1965). Eosinophilia has also been described (Welch 1943). The prompt disappearance

of the allergic state on successful treatment of giardiasis suggested that the parasite had a causal role in most of these instances.

Circulating immunoglobulins, IgE included, are usually normal in symptomatic patients with giardiasis (Jones and Brown 1974, Wright et al., 1977, Geller et al., 1978).

Changes in lamina propria plasma cells and secretory antibodies in patients with giardiasis

The demonstration that giardiasis occurs frequently in patients with hypogammaglobulinaemia (Ament and Rubin, 1972) stimulated a search for abnormalities of local immunoglobulin synthesis and secretion in the small intestine of giardiasis patients with normal serum immunoglobulins. Zinneman and Kaplan (1972) and Popovic et al., (1974) found decreased levels of IgA in small intestinal secretions, both before and after treatment in the latter study. Jones and Brown (1974) were unable to confirm these findings, but instead found raised levels of IgG in intestinal fluid from giardiasis patients. The paper by Zinneman and Kaplan (1972) provoked a letter from McLelland et al., (1973) in which they emphasized the methodological difficulties of such studies and the caution with which they should be interpreted.

Direct immunofluorescence on jejunal biopsy specimens has been used to enumerate the lamina propria plasma cells of different Ig classes. Popovic et al., (1974) studied IgA, IgM and IgG cell numbers, and showed that IgA cell numbers were reduced while IgM cells were increased in number

at the time of infection. However, Thomson et al., (1977) found raised numbers of both IgA and IgM cells, Blenkinsopp and colleagues (1978) found increases in IgA, IgM and IgG containing cells, while Ridley and Ridley (1976) described raised IgM cell numbers but normal numbers of IgG and IgA cells. They suggested that an increase in IgM cells may represent an early response to the parasite which might be followed by IgA and IgG synthesis. In none of these studies were all five classes of immunoglobulin studied.

Intraepithelial lymphocyte counts in patients with giardiasis

The presence of acute inflammatory cells in the epithelial layer was reported by Yardley et al., (1964) and Hoskins et al., (1967), but the numbers of intraepithelial lymphocytes (IEL) were not remarked upon in either study. Only one report of IEL counts in adults with giardiasis (Wright and Tomkins, 1977) and one in children (Ferguson et al., 1976) have appeared in the literature. In both cases raised IEL counts were found at the time of infection, and the numbers were found to fall following treatment in the study by Wright and Tomkins (1977). Intraepithelial lymphocytes are mainly T cells (Guy-Grand et al., 1974, Parrott and Ferguson 1974), and these observations therefore suggest that cell-mediated immunity may play an important part in the immune response to giardiasis.

Serum antibodies in humans with giardiasis

Culture of Giardia organisms in the laboratory has proved difficult, and studies of immunity in giardiasis have

therefore been hampered by the lack of suitable antigenic material in adequate quantities. Immunofluorescence studies with crude preparations of Giardia cysts isolated from patients' stools did, however, suggest that a systemic antibody of the IgG class may be found in giardiasis patients, chiefly those with malabsorption (Ridley and Ridley 1976, Vinayak et al., 1978). These authors, however, commented on the difficulty of obtaining uncontaminated cysts, and it seems likely that such cysts, culled from the patients' excretion, will be coated with secretory immunoglobulin.

Further attempts to culture the organism have met with more success (Meyer 1976, Gillin and Diamond 1980). Serological studies using these cultured trophozoites as antigen in either an indirect immunofluorescence test or an enzyme-linked immunosorbent assay have demonstrated that the majority of patients with symptomatic giardiasis produce IgG antibodies (Visvesvara et al., 1980, Smith et al., 1981). Smith et al. suggested that the enzyme-linked assay might be suitable for epidemiological surveys, and in a preliminary study demonstrated antibodies to Giardia lamblia in 14% of an apparently normal population of subjects in Washington State. However, although the serological markers may prove useful in diagnosis, the relevance of these antibodies to the host's defence mechanisms is uncertain, and further information is required on the natural history of the antibody response before the exact significance of this figure for antibody prevalence can be assessed.

HOST IMMUNE RESPONSES IN EXPERIMENTAL MURINE GIARDIASIS

Both cellular and humoral immune responses have been documented in mice experimentally infected with Giardia muris. The duration of infection has been shown to vary in different strains of mice (Roberts-Thomson and Mitchell, 1978), and mice experimentally infected develop prolonged resistance to the infection on subsequent challenge (Roberts-Thomson et al., 1976). Congenitally athymic nude (nu/nu) mice do not cure themselves of the infection until their cellular immune status is restored by transferring lymphocytes from syngeneic mice with normal immune functions and the process of parasite elimination is accelerated when the donor mice themselves have acquired protective immunity to giardiasis, (Stevens et al., 1978, Roberts-Thomson and Mitchell 1978). These studies suggest that T lymphocytes are fundamentally important to the hosts' ability to rid itself of the infection, whether indirectly by means of an antibody-mediated response requiring T cell help, or by cytotoxic effector T cells acting directly on the parasite.

The only antibody studies so far reported concern the breast milk of suckling females previously infected with Giardia muris (Andrews and Hewlett 1981). In these studies the mothers' milk was shown to contain antibodies to Giardia trophozoites of the IgA and IgG classes. This appeared to confer protection to the infants as long as they suckled, at the expense of the mothers, who became

susceptible to reinfection with Giardia (Stevens and Frank 1978), but the authors admitted that immunity may have been transferred by primed, gut-derived lymphocytes as well as by specific antibody.

An indication that circulating antibodies could be important comes from a recent study in which rabbit peritoneal macrophages from animals not previously exposed to Giardia phagocytosed G. muris trophozoites much more effectively in the presence of serum from previously infected mice than in the presence of normal serum, suggesting that antibodies present in the immune serum might have an opsonising effect (Radulescu and Meyer 1981). The relevance of this to the normal method of parasite expulsion is, however, still far from clear.

Owen et al., (1979) used the scanning electron microscope to examine the relationship between Giardia trophozoites and the host intestine in experimentally infected mice. They found that the trophozoites were present in greatest numbers in the proximal 25% of the small intestine, and tended to adhere to the microvilli of columnar cells near the bases of villi. The organisms did not attach to the M cells of Peyer's patches, these being the cells specially adapted for the absorption of intact soluble antigens, but did gain access to the Peyer's patch tissues by means of defects in the overlying columnar epithelium. In addition, they recorded the presence of lymphoid cells within the intestinal lumen during the expulsion phase of the infection, and indeed

these lymphocytes were seen to adhere to the dorsal surfaces of Giardia trophozoites. It therefore seems likely that effector T cells do cross the epithelium to attack the parasite within the lumen. Using transmission electron microscopy Owen and his colleagues have gone on to show in a series of beautiful photographs that trophozoites finding their way into epithelial breaches are engulfed by macrophages which extend pseudopodia through the epithelial basement membrane. The trophozoite is then digested within a lysosome in the macrophage cytoplasm, and the macrophage is surrounded by small lymphocytes. It is presumed that processed antigen is then transferred to the small lymphocytes, which will undergo blast transformation before homing to the mucosa via the thoracic duct (Owen et al., 1981).

In a detailed set of experiments to examine the reasons for the failure of C3H/He mice to eliminate the parasite, Underdown et al., (1981) uncovered a paradox. It appears that this strain of mouse, although unable to rid itself of a primary infection, nonetheless acquires specific protective immunity to subsequent infection when cured by treatment with metronidazole. The kinetics of acquisition of this immunity, as judged by the ability to transfer immunity to suckling young, did not differ from that observed in BALB/c mice which eliminate the parasite rapidly. The authors suggested that the explanation for this might not relate to any abnormality of host immunity, but rather to the hosts' relative inability to counteract

a putative substance which may be secreted by the parasite in an attempt to suppress the hosts' local immune defence mechanisms. This is clearly highly speculative.

In summary it is plain from the animal studies and from the finding of raised IE lymphocyte counts in humans that T cells are important in the intestinal immune response to giardiasis. Antibody responses have been shown to occur in both humans and animals, but their significance to host immunity is not clear and there is particular controversy surrounding the contribution of mucosal antibody-synthesising cells. The timing of these immune reactions in relation to the course of infection and the evolution of pathological changes in the small intestine has not been studied, and forms the subject of this thesis.

CHAPTER 3
MATERIALS AND METHODS

1. STUDIES OF GIARDIASIS IN HUMANS

Chapter 4 consists of a description of patients with giardiasis whom I have seen while working in a gastro-intestinal unit. Full details of these patients and of the investigations performed are contained within that chapter.

Immunofluorescence method for the counting of mucosal immunoglobulin-containing cells in humans with giardiasis

Jejunal biopsies were taken just beyond the ligament of Treitz by Crosby capsule, and were immediately divided into 2 portions. One portion was fixed conventionally in Bouin's solution and stained with haematoxylin and eosin for histological examination, while the second portion was processed for direct immunofluorescence using the method of Brandtzaeg (1974). Monospecific antisera for each of the 5 immunoglobulin classes were supplied by Behringwerk A.G. Germany. All sera were diluted 1 : 20 before use. The specificity of the anti-epsilon serum was verified by immunoelectrophoresis and Ouchterlony double immunodiffusion against both normal plasma protein and myeloma IgE kindly supplied by Dr. J.P. Vaerman (Experimental Medicine Unit, Louvain University, Belgium). The immunofluorescence reactions were checked by absorption studies including immunoadsorption of anti-IgE serum with the IgE myeloma protein. The Ig-containing cells of each class were enumerated by counting the numbers

of fluorescent cells in 3 adjacent tissue units of 0.24 x 0.37 mm, as measured by an eyepiece graticule aligned along the tips of the villi. This method was an adaptation of the technique described by Brandtzaeg et al., (1974). The counts were formed on a coded basis by one observer. Figures 3.1 and 3.2 show typical examples of specimens stained for IgA and IgE respectively.

Intraepithelial lymphocyte counts in humans with giardiasis

The method used for counting intraepithelial lymphocytes was the same for both human and mouse studies. Biopsies were fixed in either formalin or Bouin's solution and stained with haematoxylin and eosin. Differential cell counts were performed on well orientated parts of the biopsies and the results expressed as the number of lymphocytes per 100 epithelial cells. A minimum of 500 epithelial cells were counted per biopsy.

2. STUDIES USING THE ANIMAL MODEL OF GIARDIASIS

Animals

For all experiments performed in the U.K. CBA mice of both sexes were used at the age of 2 - 3 months. They had free access to tap water and pelleted rodent diet (Stratt's mouse pellets providing 4.2 calories/gram, protein content 21.3%). In France BALB/c mice of both sexes were used at age 2 - 3 months and were maintained under similar conditions, receiving UAR mouse pellets (UAR, Villemoisson-sur-

Orge, France). Faecal specimens and intestinal contents of stock mice were checked regularly to confirm the absence of protozoal and helminth infections in the colonies.

Giardia muris infection

Giardia muris cysts were provided by Dr. I. Roberts-Thomson and flown from Australia to the United Kingdom by airmail. Stock mice were infected by oral inoculation of 1,000 cysts, and the infection maintained by weekly inoculation of two or three adult mice. For all experiments, mice aged 8 - 10 weeks were infected by intragastric administration of 1,000 Giardia muris cysts in 0.2 ml tap water. Cysts were isolated and counted by the method of Roberts-Thomson et al., (1976) described below.

Cyst counts

A two hour faecal collection was obtained from each mouse by isolating it within a plastic tub for two hours. Faeces were then broken up in tap water and the faecal suspension layered on molar sucrose of specific gravity 1.11, and centrifuged at 400 G for 15 minutes. Cysts, concentrated at the water-sucrose interface, were removed, washed in normal saline and resuspended in a known volume before counting in a haemocytometer. With this technique the limit of detection is 1,000 cysts per specimen.

Trophozoites - histology

In animals which were used for measurements of epithelial cell kinetics and disaccharidases, the presence of trophozoites in the intestine was confirmed histologically or by direct smear of jejunal contents.

Trophozoite counts - vibration method

A new method for isolating and counting giardia trophozoites was developed in order to make possible the accurate assessment of the magnitude of infection at any given time; it was considered to be of fundamental importance for the interpretation of many of the experiments described in this thesis. This technique also provided a suspension of trophozoites as antigen in the immunofluorescent antibody studies described in Chapter 9. The details of the technique, the methods used to verify its accuracy, and the new light it shed on our knowledge of the natural history of the infection in mice are described in Chapter 6.

Trophozoite count - sieve method

In order to define the distribution of trophozoites along the intestine, another technique was used in a small number of animals. The whole small intestine was removed and its length measured. Beginning at the gastro-duodenal junction, segments of whole intestine 1 cm in length were taken, discarding 2 cm between each. Thus counts were performed on one third of the entire intestine. Each 1 cm

segment was mashed through a stainless steel grid with holes of 50 μ m diameter, using a glass rod, and washed through with 1 ml normal saline from a syringe and needle. Aliquots of the resulting suspension were then examined and a trophozoite count performed in a Neubauer haemocytometer.

Measurements of epithelial cell kinetics

A stathmokinetic technique, using colchicine blockage and microdissection was used. A group of mice (10 - 15 in most experiments) was taken and colchicine (BDH) injected, 5 mg/kg body weight, intraperitoneally. Mice were killed by ether overdosage or cervical dislocation at intervals from 30 minutes to 2½ hours later and a piece of jejunum, 10 cm from the pylorus, and a piece of ileum, 5 cm from the ileo-caecal valve were dissected out and fixed in a 75% absolute alcohol, 25% glacial acetic acid mixture, for 24 hours. Tissues were then transferred to 75% alcohol until processed for measurements of villus length, crypt length and metaphases/crypt.

In order to count metaphases, a fragment of the tissue was placed on a slide in 45% acetic acid and gently squashed with a coverslip. The number of metaphases/crypt was counted. Metaphase blockage was found in every specimen examined. For each specimen the number of metaphases in 10 crypts was counted and the mean value was obtained. The crypt cell production rate was calculated from the slope of the regression line, drawn

using the method of least squares, of metaphase count against time after colchicine injection.

Measurements of intestinal architecture

For measurements of crypts and villi, tissues processed as described above were stained in bulk by an adaptation of the Feulgan technique of Wimber and Lamerton (1963). The tissues were stained as follows: 50% alcohol 10 minutes; tap water 10 minutes; hydrolysis in molar HCl at 60°C 6 minutes; tap water 10 minutes; Schiff reagent 20 minutes; then stored in tap water until examined. With a dissecting microscope the tissue was orientated and one or two villi with their surrounding crypts were cut from the edge by careful dissection with a scalpel and fine forceps, placed on a slide in 45% acetic acid, and lengths of villi and crypts measured by a calibrated micrometer eyepiece. For each specimen ten measurements of villus length and of crypt length were made and the mean value obtained.

Histology and intraepithelial lymphocyte counts

At the same time as specimens were taken for epithelial cell kinetic measurements, adjacent segments of jejunum and ileum were fixed in formol saline and paraffin embedded, 5 μ m sections were cut and stained with haematoxylin and eosin. Intraepithelial lymphocyte counts were performed by a differential count in the epithelium covering the villi, in well orientated H and E

stained sections. Results were expressed as lymphocytes/100 epithelial cells. In addition, an assessment of the heaviness of Giardia infection was made, scoring each section as containing many trophozoites (confluent sheets of trophozoites in the mucus over the villi), few trophozoites (single organisms, at least one or two per villus, and readily seen) or no trophozoites (no parasites seen in six to eight high power fields).

Disaccharidase assay

Tissues for disaccharidase assay were taken from jejunum around 10 cm from the pylorus, and ileum, 5 cm from the ileo-caecal valve. The full thickness specimen of intestine was weighed, homogenised in ice-cold maleate buffer, and the disaccharidases lactase, sucrase and maltase assayed by the technique of Dahlquist (1968).

Measurement of circulating anti-Giardia antibodies in the mouse

Antigen

The vibration method for measuring trophozoites described in detail in Chapter 6 was adapted to provide a source of antigen for these immunofluorescence studies. Animals infected between 2 and 5 days previously were used in the hope that this would provide trophozoites unaffected by secretory antibodies from the host animal. The initial vibration time of the whole everted small intestine in normal saline was restricted to 2 minutes to avoid as far as possible contamination of the suspension with intestinal

debris, mucus and epithelial cells, and acetyl cysteine was not included in the medium. The suspension of trophozoites was centrifuged as described in Chapter 6, and then diluted to a final concentration of approximately 5×10^4 trophozoites/ml. Aliquots of 10 μ l of this suspension were allowed to dry on microscope slides degreased with alcohol, heat-fixed and processed for immunofluorescence as described below. For each experiment the same antigen suspension was used for control and test sera. Fig. 3.3 shows fixed, unstained trophozoites.

Antisera

Goat anti-mouse IgA and IgM were provided by Dr André, who had prepared these antisera for previous experiments. Rabbit anti-mouse IgG (pooled to include subclasses IgG1, 2a, 2b and 3) was obtained from Bionetics Laboratories (London). For IgA and IgM studies fluorescein-coupled rabbit anti-goat immunoglobulin was used, and fluorescein-coupled goat anti-rabbit immunoglobulin for the IgG studies, the concentration of fluorescein-conjugated immunoglobulin being in each case around 10 mg/ml (antisera supplied by Nordic Immunological Laboratories).

Immunofluorescence method

Indirect immunofluorescence was employed using doubling dilutions of test sera from 1 : 2 to 1 : 128. 10 μ l of the serum to be studied was placed on a fixed

trophozoite antigen preparation as described above, and incubated at room temperature in a humid atmosphere for 30 minutes. The slides were then washed in physiological saline for increasing periods of time (5, 10, 15, 20 and 25 minutes). They were then carefully wiped dry and 10 μ l of the respective anti-mouse immunoglobulins, diluted 1 in 10, were applied and again incubated at room temperature in a moist atmosphere for 30 minutes. They were again washed in successive baths of physiological saline for 5, 10, 20, 30 and 30 minutes. The slides were again wiped dry and the appropriate fluorescein-conjugated antisera, diluted 1 in 15, were applied in 10 μ l aliquots. After washing, using the same lengths of time as for the first step above, the results were read immediately using a Dialux fluorescence microscope equipped with a Ploem illuminator and mercury vapour lamp. The reciprocal of the highest dilution of test serum showing definite fluorescence was taken as the antibody titre. Fig. 3.4 shows a negative control and positive fluorescence for IgG.

Statistical analyses

In the studies of giardiasis in humans described in Chapter 4 and 5, all comparisons were made using Student's t test.

In most of the animal experiments described in Chapter 6 - 9 multiple comparisons were made between groups studied at different times during the course of the infection. All such results were first analysed for significant differences using parametric (F test) and

non-parametric (Kruskall-Wallis) one way analyses of variance. Where appropriate the differences between individual groups were then assessed using Student's t test.

In Chapter 7 the results of the experiment measuring crypt cell production rate were analysed by the method of least squares.

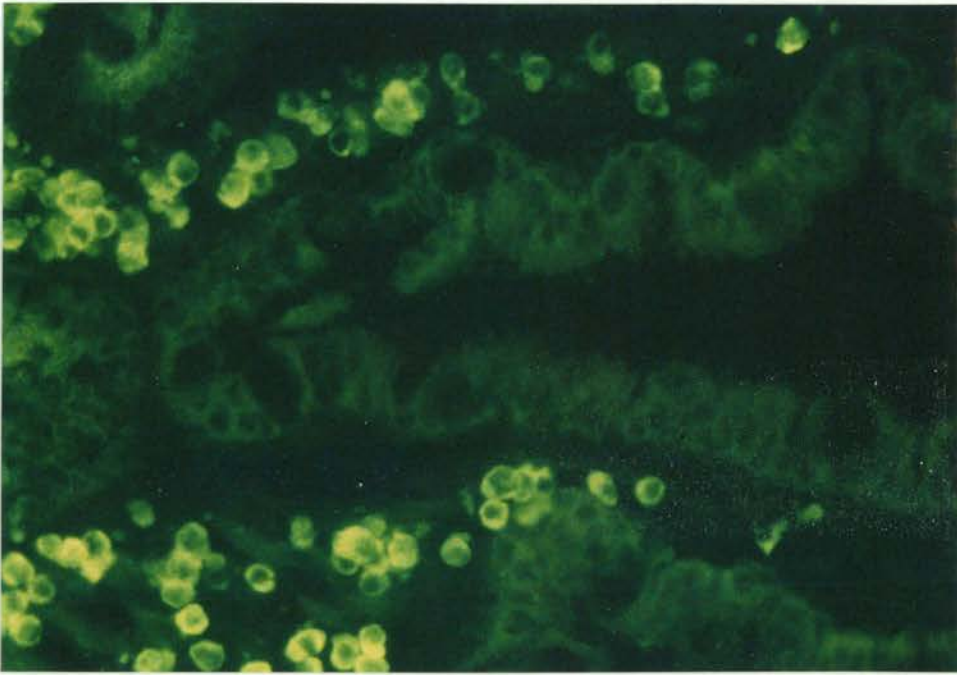


Figure 3.1 Jejunal biopsy from a patient with giardiasis, stained by indirect immunofluorescence for IgA (x400).



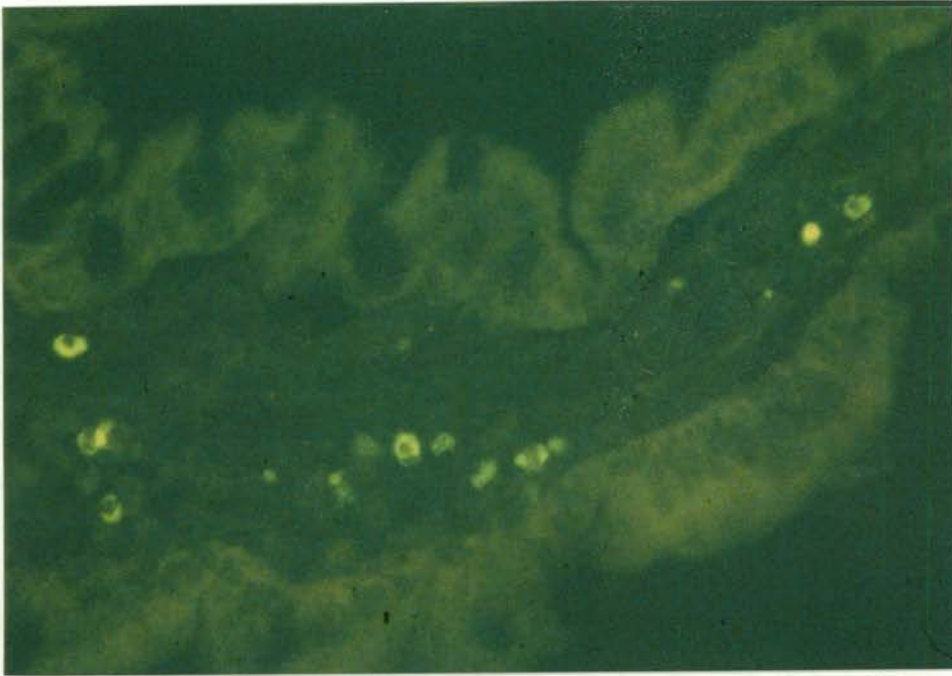


Figure 3.2 Jejunal biopsy from a patient with giardiasis, stained by indirect immunofluorescence for IgE (x400).



Figure 3.3 Photomicrograph of fixed, unstained trophozoites prior to processing for immunofluorescence (x400).



Figure 3.4 (a) Trophozoites showing negative immunofluorescence for IgG - normal control serum (x250).

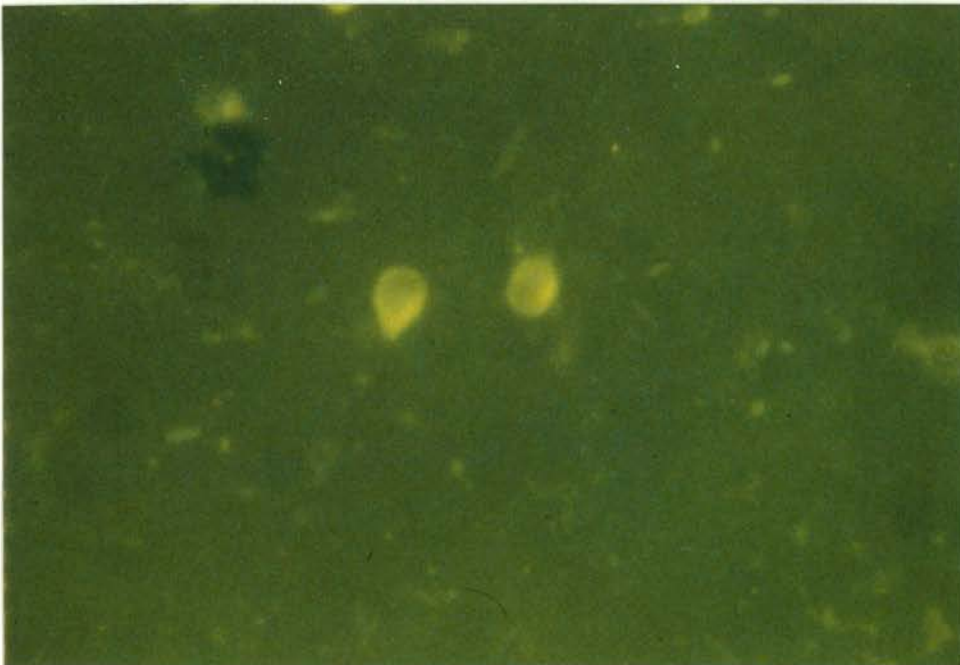


Figure 3.4 (b) Positive immunofluorescence test for IgG using serum from a previously infected mouse (x400).

CHAPTER 4

CLINICAL STUDIES IN ADULTS PRESENTING WITH GIARDIASIS TO A GASTRO-INTESTINAL UNIT

INTRODUCTION

In spite of the fact that sporadic cases, and occasionally epidemics, of giardiasis are known to occur in temperate zones (Petersen 1972, Meuwissen et al., 1977, Meyer and Jarroll 1980) all of the studies of adult patients in the UK have focused on giardiasis in travellers returning from areas where the disease is endemic. There is therefore little information on the relevance of giardiasis to adult clinical practice in this country. Although prevalence rates have been calculated for several temperate countries (Petersen 1972, Quinn 1971), these have generally relied on faecal cyst detection for diagnosis, and are therefore likely to underestimate the true incidence of the disease, accurate assessment of which will only be achieved when more sensitive screening methods, such as serology, can be made more generally available. Similarly, the number of sporadic cases identified in clinical practice is likely to be falsely low, since it is likely that the diagnosis is often missed when stool examination is the only investigation performed (Ament and Rubin 1972, Kamath and Murugasu 1974) and the usually self-limiting nature of the infection (Rendtorff 1954) makes it likely that only occasional patients with severe or chronic symptoms will be subjected to further investigations such as jejunal aspiration and biopsy.

Thus, although giardiasis is probably not uncommon, it is relatively infrequently seen in adult hospital practice

in this country. I therefore set out to document all cases of giardiasis identified in a gastro-intestinal unit during a defined period of time. These patients were studied in detail in order to compare the clinical and laboratory findings with published series, and to try to identify any factors either predisposing to the infection or likely to lead to chronicity.

PATIENTS AND METHODS

During a 2 year period 8 patients were identified as having giardiasis using existing investigative procedures in an adult gastro-intestinal unit. In this unit approximately 1000 new patients are seen per annum, and approximately 180 jejunal biopsies are performed each year.

A history was obtained from each patient, including a record of foreign travel and any other relevant disease or previous surgery, and a full physical examination carried out. In most instances stools were examined for cysts of Giardia lamblia prior to further investigation, but in 3 cases the diagnosis was unsuspected, being made by jejunal biopsy done in the investigation of non-specific symptoms. The protocol for investigation of these patients is outlined in Table 4.1.

The diagnosis was established in 6 patients by peroral jejunal biopsy with a Watson capsule, and in 2 patients by endoscopic biopsy. As is routine practice in the

unit, part of each Watson capsule biopsy was submitted for estimation of disaccharidase content, the remaining portion being processed for histology, and for measurements of villus height and crypt depth. These measurements of mucosal architecture were performed on the H and E stained specimens using a calibrated microscope eyepiece. Ten counts of villus height, crypt depth and epithelial cell height were made on each specimen and the means calculated. For control values, counts were performed on 10 subjects who had undergone jejunal biopsy either as normal controls in the course of another research project or in the investigation of symptoms for which no organic cause was found. Disaccharidase studies were not performed in the 2 patients diagnosed at endoscopy.

Blood was taken for haemoglobin, differential white cell count, liver function tests, plasma protein electrophoresis and serum immunoglobulin levels. Tests of absorption included a Schilling test without intrinsic factor and faecal fat estimation. Breath H_2 excretion in response to an oral glucose load was measured in 5 patients as an index of bacterial activity in the small bowel. As many as possible of these investigations were repeated in the 6 patients in whom follow-up studies could be performed (one patient left the area and one defaulted from follow-up after initial investigations and treatment). The repeat studies were carried out within one month of completing a 10 day course of metronidazole 400 mg qds.

RESULTS

Patients

Giardiasis was diagnosed in 5 females and 3 males (age range 25 - 52) during the 2 year period of the study. The details are summarised in Table 4.2. All but one were caucasians native to the UK, the exception being a Pakistani lady who had come to Scotland 3 years previously. Four patients gave a history of travel to tropical or sub-tropical countries shortly before presenting with giardiasis, the countries visited being Iran, the Philippines, India and Pakistan. The patient who seemed to have contracted the disease in the Philippines had been resident there for some years and had chronic symptoms.

Clinical features

Diarrhoea was a major presenting symptom in only 5 of the 8 patients. Two complained of abdominal pain, 3 had suffered from recurrent headaches, and 2 had noticed significant loss of weight. Patient 2 had suffered recurrent anaemia for many years, during virtually all of the time she had been resident in the Far East. Patient 3 was being investigated for multiple symptoms and iron deficiency anaemia following gastroenterostomy for duodenal ulceration. None of the patients presented the clinical picture of "malabsorption syndrome". In no case did physical examination contribute any additional information.

Laboratory investigations

The results of initial laboratory investigations are summarised in Table 4.3. With the exception of patient 3 all of the patients had normal haemoglobin levels at the time of the study. Patient 8 had an eosinophilia of 8% before treatment, falling to 4% after metronidazole. She had no symptoms of allergy.

Routine clinical chemistry screening, including liver function tests and serum calcium, was invariably normal. Serum immunoglobulin levels were normal in all except one patient (patient 8), who had a persistently low level of IgA (48 IU/ml).

Vitamin B₁₂ metabolism

Serum levels of vitamin B₁₂ were invariably normal. Of the 7 patients who had Schilling tests prior to treatment, only one fell within the normal range, the others showing mild to moderate impairment (Table 4.4). Three of those with moderately severe defects in B₁₂ absorption (patients 1, 5 and 7) had repeat studies after treatment, and in all 3 vitamin B₁₂ absorption had returned to normal (6.9%, 7.7% and 10.6% before, 32.3%, 31.8% and 31.1% after, respectively). Patient 5 had previously had a partial gastrectomy, and therefore was given intrinsic factor on each occasion.

Faecal fat excretion

Three day faecal fat excretion was measured in 7 patients (Fig. 4.1). Only one patient (patient 4) had an unequivocally

normal result. The two highest values occurred in the patients who had previously had gastric surgery (patients 3 and 5, 27.5 and 32.1 mmol/24 hrs respectively). The other patients exhibited only marginally elevated faecal fat excretion (range 18 - 24 mmol/24 hrs, normal < 18).

Repeat values after treatment were only obtained in 2 patients (patients 1 and 8). The results before and after treatment were 20.8 and 16.3 mmol/24 hrs (patient 1) and 24 and 13 mmol/24 hrs (patient 8). Thus in both cases a slightly raised faecal fat excretion returned to normal with eradication of the parasite.

Breath hydrogen excretion (Hepner 1974)

None of the 5 patients tested had abnormal levels of hydrogen in the breath while fasting. After ingestion of 50 g glucose patient 3 had an early increase in H₂ excretion (fasting - 5 mmol/l, 15 minutes - 5 mmol/l, 30 minutes - 14 mmol/l, 45 minutes - 34 mmol/l), but this was probably attributable to early gastric emptying and rapid transit resulting from his previous gastroenterostomy and vagotomy rather than microbial activity in the small intestine. Normal patterns were seen in all other patients tested.

Jejunal histology

Giardia lamblia trophozoites were identified in all 8 jejunal biopsies stained with haematoxylin and eosin. The histological findings are summarised in Table 4.5. Four

patients (patients 3 - 6 inclusive) had entirely normal mucosal architecture. Patients 1 and 2 exhibited partial villus atrophy with a mixed inflammatory infiltrate, and patient 8 had villi slightly shorter than normal with increased numbers of lymphocytes and plasma cells in the lamina propria.

Measurements of villus height, crypt length and epithelial cell height showed no significant difference between normal controls and giardiasis patients, nor between the patients before and after treatment, for any of the 3 parameters (students' t test) (Table 4.6). Giardia patients, however, had a lower mean villus height and greater crypt length than controls, and after treatment had a greater mean villus height than controls. These changes are similar to those found in mice (Chapter 7), and may have achieved statistical significance had greater numbers of patients been available for study.

Patient 7 had a pronounced mixed inflammatory infiltrate but had normal mucosal architecture. The villi were confirmed to be normal in appearance on scanning electron microscopy, and this technique also confirmed the presence of a heavy trophozoite load (Fig. 4.2). After treatment patients 1 and 7 had normal jejunal biopsies, while patient 2 still had mild partial villous atrophy. A repeat biopsy was not obtained from patient 8, nor from patients 3 and 6. Patients 4 and 5 had normal biopsies before and after treatment. In all of the repeat biopsies the absence of trophozoites was confirmed.

Disaccharidases in small intestinal mucosa

Six patients had disaccharidase levels measured at the time of the first jejunal biopsy. Patient 4 had entirely normal levels of all 4 enzymes (lactase, maltase, sucrase and trehalase). Patients 1 and 2 had reduced levels of all 4, and in patient 2 completely normal results were obtained after treatment. Patient 1 had a persistently very low level of lactase even after treatment, the other 3 enzymes having reverted to normal. He reported that his lactase intolerance had persisted, and required a lactose-free diet. Patient 7 had a slightly low lactase level which reverted to normal after treatment. Patient 6 had a reduced level of sucrase and patient 8 a slightly reduced lactase, but in neither of these was it possible to repeat the test after treatment. The lactase levels prior to treatment are shown in Fig. 4.3.

FACTORS PREDISPOSING TO GIARDIASIS OR TO FAILURE OF PATIENTS TO ERADICATE THE PARASITE

Rendtorff (1954) clearly demonstrated that giardiasis in adults is usually self-limiting and frequently asymptomatic. It might therefore be expected that most patients presenting for hospital investigation of relatively severe or chronic symptoms will have some characteristic which renders them more prone to infection or less able to effect self-cure. Of the 8 patients identified in this study

4 had probably contracted the disease in tropical or sub-tropical countries where it is endemic. One of these patients, a 42 year old Pakistani female, had previously been diagnosed as having sarcoidosis, a disease in which cell mediated immunity (CMI) is impaired. Roberts-Thomson and Mitchell (1978) have shown that impaired CMI leads to prolonged infection in mice experimentally infected with giardia, and it is therefore possible that this patient had chronic giardiasis as a consequence of her sarcoidosis. She was not receiving corticosteroids at the time of the study. The other 3 patients contracting the disease abroad had no obvious predisposing factors.

Of the 4 patients with no history of foreign travel, 2 had previously had gastric surgery and one had persistently low serum levels of IgA. It has been suggested that gastric surgery may predispose to giardiasis (Yardley et al., 1964, Giannella et al., 1973, Vachon et al., 1963). The numbers of reported cases are small, and the present study therefore supports this concept. However, the mechanism is far from clear, the assumption by previous authors that absence of the gastric acid barrier increases susceptibility being difficult to accept, since the parasite normally causes infection in the presence of intact gastric acid secretion and may even depend on gastric acid for excystation (Bingham and Meyer 1979).

Although there is no doubt that hypogammaglobulinaemia predisposes to symptomatic giardiasis, frequently in a severe form, (Ament and Rubin, 1972), the relationship

between selective IgA deficiency and giardiasis is less clear (Kraft 1979, Jones and Brown 1974) although sporadic cases have been reported (Hoskins et al., 1967, Zinneman and Kaplan 1972). The fact that one of the 8 patients in this study had a modest degree of IgA deficiency, the commonest immunodeficiency in the population is therefore likely to be coincidental, particularly in view of the fact that the degree of IgA deficiency was slight in comparison with most patients exhibiting selective IgA deficiency.

SUMMARY


Eight adults presenting with giardiasis to a gastrointestinal unit during a 2 year period were studied in detail. Symptoms were varied, diarrhoea occurring in only 5 patients. Four of the 8 gave a history of travel to endemic zones, and 3 of those who gave no such history had possible predisposing conditions (gastric surgery [2], and borderline IgA deficiency [1]). Most patients exhibited mild malabsorption of fat and vitamin B₁₂, and some abnormality of jejunal disaccharidases was usual. Jejunal histology varied from normal to partial villus atrophy with a variable infiltrate of mixed inflammatory cells. No statistically significant differences in villus height, crypt length or epithelial cell height could be detected between giardiasis patients and controls, nor between patients before

and after treatment. Treatment with metronidazole was uniformly successful, and most of the above abnormalities reverted to normal within a month.

TABLE 4.1

PROTOCOL FOR INVESTIGATION OF GIARDIASIS

Stool microscopy

Jejunal biopsy  Pathology (including IEL count)
Disaccharidases

Hb, WBC and differential

B₁₂, folate

Immunoglobulins

Schilling test without IF

Faecal fat

Breath H₂

Tests to be repeated 1 month after 10-day course
of Metronidazole 400 mg QID

TABLE 4.2

CLINICAL FEATURES OF THE PATIENTS STUDIED

| <u>PATIENT</u> | <u>AGE</u> | <u>SEX</u> | <u>NATIONALITY</u> | <u>SYMPTOMS</u> | <u>TRAVEL</u> | <u>OTHER FACTORS</u> |
|----------------|------------|------------|--------------------|--|---------------|-----------------------------|
| 1 | 46 | M | British | Diarrhoea, headaches | Iran | - |
| 2 | 39 | F | British | Recurrent anaemia, weight loss, headaches | Philippines | - |
| 3 | 52 | M | British | Anaemia, multiple symptoms including headache | - | Gastroenterostomy for DU |
| 4 | 36 | F | British | Diarrhoea, weight loss | - | - |
| 5 | 38 | M | British | Epigastric pain, diarrhoea | - | Partial gastrectomy |
| 6 | 25 | F | British | Diarrhoea | India | - |
| 7 | 42 | F | Pakistani | Headache, abdominal pain | Pakistan | Sarcoid |
| 8 | 30 | F | British | Diarrhoea | - | - |

TABLE 4.3

INITIAL LABORATORY INVESTIGATIONS IN PATIENTS WITH GIARDIASIS

| <u>PATIENT</u> | <u>STOOL MICROSCOPY</u> | <u>HAEMOGLOBIN</u> | <u>IMMUNOGLOBULINS (IU/ml)</u> | | |
|----------------|-----------------------------|--------------------|--------------------------------|------------|------------|
| | | | <u>IgG</u> | <u>IgA</u> | <u>IgM</u> |
| 1 | Positive (1 out of 6) | 14.1 | 132 | 148 | 104 |
| 2 | Negative | 12.1 | 84 | 124 | 104 |
| 3 | Not done | 10.1 | 118 | 100 | 74 |
| 4 | Not done | 12.9 | Not done | | |
| 5 | Positive | 14.2 | 155 | 188 | 253 |
| 6 | Negative | 12.9 | 120 | 104 | 110 |
| 7 | Not done | 12.7 | 153 | 237 | 61 |
| 8 | Positive | 12.9 | 152 | 48 | 52 |

TABLE 4.4

VITAMIN B₁₂ ABSORPTION IN PATIENTS WITH GIARDIASIS

% RECOVERY IN URINE
(N > 15%)

| <u>PATIENT</u> | <u>BEFORE TREATMENT</u> | <u>AFTER TREATMENT</u> |
|----------------|-------------------------|------------------------|
| 1 | 6.9 | 32.3 |
| 2 | Not done | Not done |
| 3 | 13.2 | Not done |
| 4 | 11.5 | Not done |
| 5 | 7.7 | 31.8 |
| 6 | 9.6 | Not done |
| 7 | 10.6 | 31.1 |
| 8 | 36.9 | 28.6 |

TABLE 4.5

JEJUNAL HISTOLOGY IN 8 PATIENTS WITH GIARDIASIS

| <u>PATIENT</u> | <u>BEFORE TREATMENT</u> | <u>AFTER TREATMENT</u> |
|----------------|---|------------------------|
| 1 | PVA | N |
| 2 | PVA | Mild PVA |
| 3 | N | Not done |
| 4 | N | N |
| 5 | N | N |
| 6 | N | Not done |
| 7 | Mixed inflammatory infiltrate | N |
| 8 | Mild PVA, dense inflammatory infiltrate | N |

PVA - partial villus atrophy

TABLE 4.6

MORPHOLOGICAL MEASUREMENTS IN THE SMALL INTESTINE
OF GIARDIASIS PATIENTS AND OF NORMAL CONTROLS

| | CONTROLS n = 10 | GIARDIASIS BEFORE TREATMENT n = 6 | GIARDIASIS AFTER TREATMENT n = 6 |
|--|--------------------|---|--|
| Villus height (μ) | 380.5 \pm 59.0 | 335.2 \pm 64.3 | 403.3 \pm 98.1 |
| Crypt length (μ) | 127.1 \pm 32.4 | 153.8 \pm 31.4 | 138.6 \pm 30.5 |
| Epithelial cell height (μ) | 29.3 \pm 4.1 | 25.6 \pm 3.5 | 29.1 \pm 2.4 |

(Results expressed as mean \pm SEM)

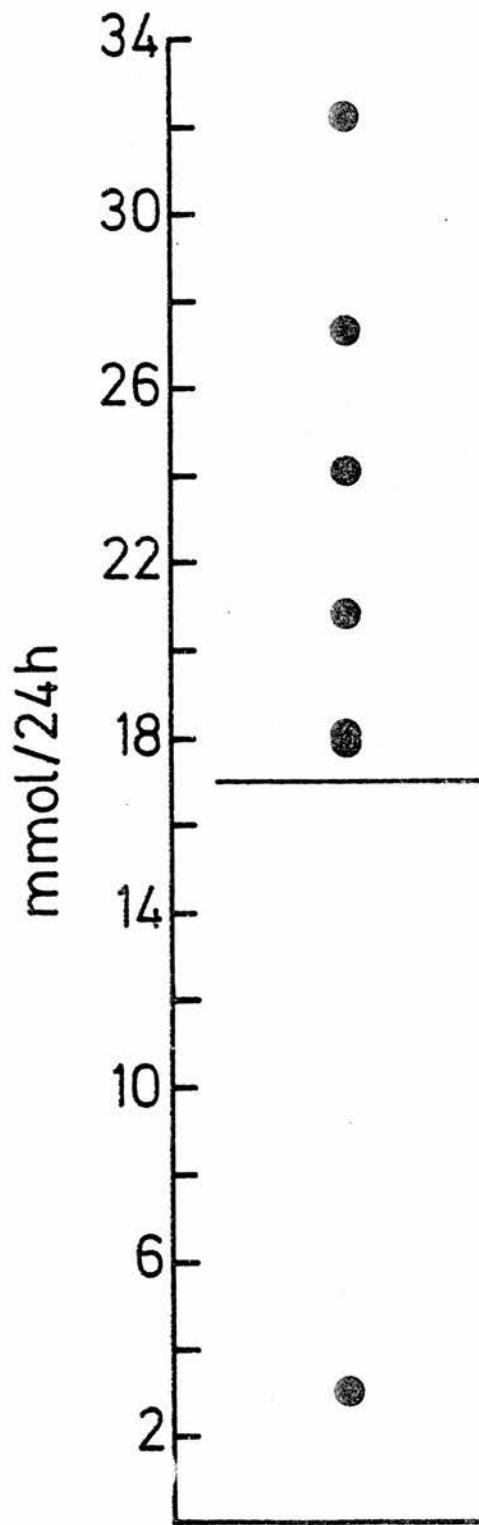


Figure 4.1 3 day faecal fat excretion in 7 subjects with giardiasis ($N < 17$ mmol/24 hrs)

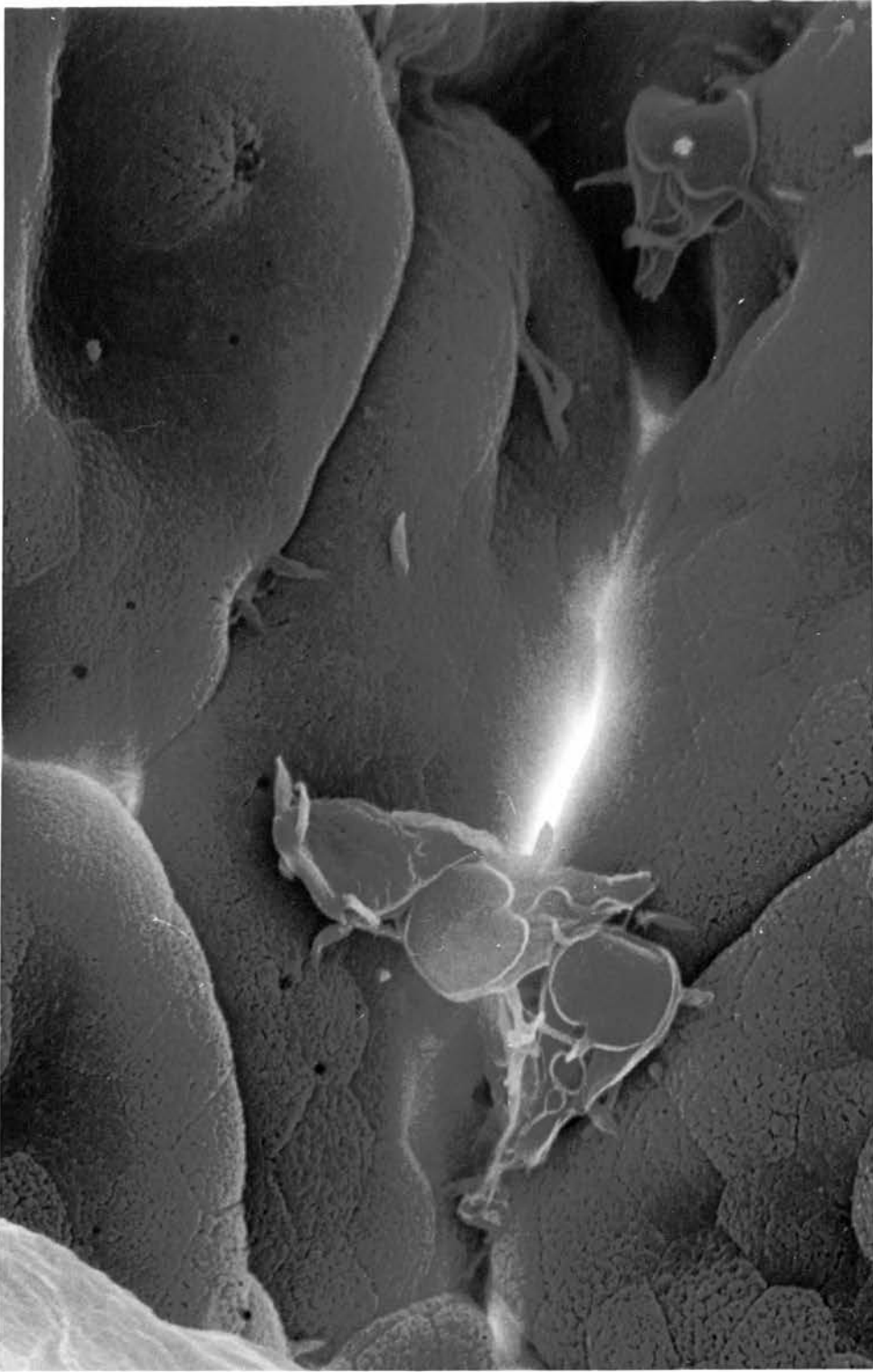


Figure 4.2 Scanning electron micrograph of jejunal biopsy from patient 7.

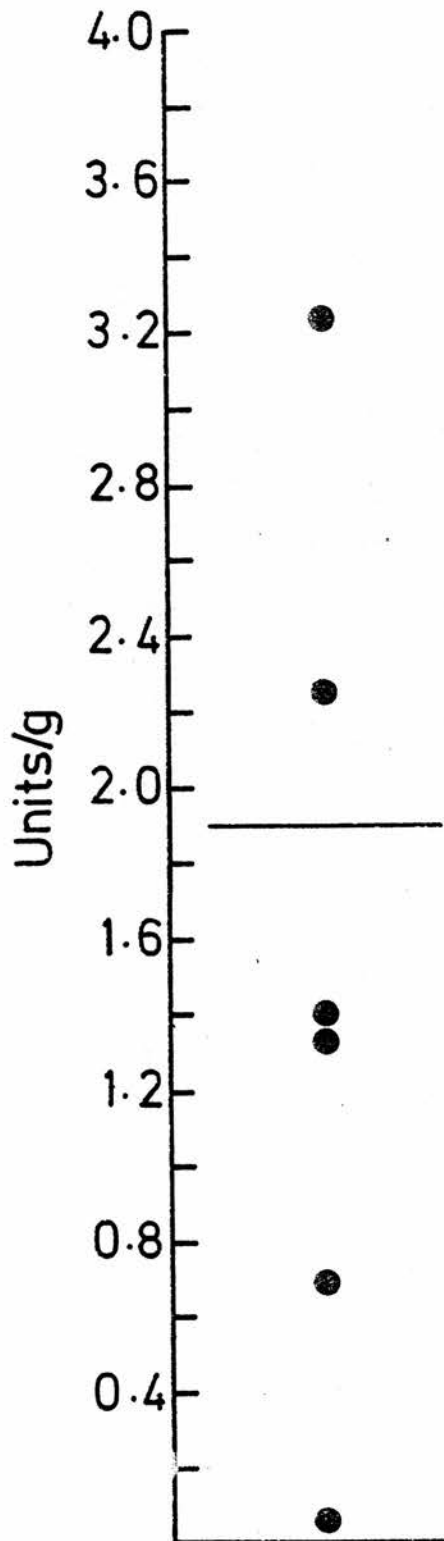


Figure 4.3

Jejunal lactase levels in 6 patients with giardiasis ($N > 1.9$ units/g)

CHAPTER 5

STUDIES OF MUCOSAL IMMUNOCYTES IN THE SMALL INTESTINE
OF HUMANS WITH GIARDIASIS

1. CHANGES IN MUCOSAL IMMUNOGLOBULIN-CONTAINING CELLS IN PATIENTS WITH GIARDIASIS BEFORE AND AFTER TREATMENT

The immunohistochemical study of jejunal biopsies from 20 adult patients with giardiasis described in this chapter was carried out while I was working in the laboratory of Dr. Claude André in Lyon, France. Clinical responsibility for these patients remained with the referring physicians, to whom I am grateful for their cooperation in allowing me access to the biopsy material.

Several studies of mucosal immunoglobulin-containing cells in giardiasis have been reported (Popovic et al., 1974; Thomson et al., 1977; Blenkinsopp et al., 1978), but results have been conflicting and none of these studies covered all five immunoglobulin classes. The aim of the present study was to examine the numbers of cells of all five immunoglobulin classes in a larger number of patients that had previously been used, and to assess the effect of successful treatment of the infection on immunocyte numbers.

PATIENTS AND METHODS

Twenty adult patients with symptomatic giardiasis were studied. Symptoms ranged from mild abdominal discomfort to diarrhoea with or without malabsorption. Circulating immunoglobulin levels were normal in all patients. The diagnosis was confirmed by jejunal biopsy

in all cases, both histologically and by making a direct smear from the biopsy. None of the patients had significant villus atrophy. Metronidazole 1 g daily was prescribed as two 5-day courses separated by an interval of one week. All patients reported symptomatic improvement after treatment, and 10 consented to a further jejunal biopsy. These 10 patients had their second jejunal biopsy performed 1 month after the start of the first course of treatment.

The immunofluorescence and cell counting methods are described in Chapter 2.

RESULTS

Before treatment with metronidazole patients with giardiasis had total numbers of Ig-containing cells slightly greater than controls, but this difference was not statistically significant. However, the relative proportions of the 5 Ig classes differed markedly from controls, the most significant abnormalities being an increase in IgE-containing cells (patients 22 ± 5 vs. controls 2 ± 0.5 , $p < 0.001$), and IgD-containing cells (patients 7 ± 2 , controls 2 ± 0.5 , $p < 0.005$). Giardiasis patients had lower numbers of IgA cells and greater numbers of IgM cells, but these differences failed to reach statistical significance (Student's t test for unpaired data). No difference was observed in the IgG-containing cell numbers. These results are summarised in Table 5.1.

The results for the 10 patients who had a second biopsy after treatment are shown in Table 5.2. All 10 patients reported symptomatic improvement after treatment, and no giardia trophozoites were found in any of the repeat biopsies. Before treatment the total numbers and relative proportions of Ig-containing cells did not differ from the total group of 20 patients. After treatment there was a significant decrease in IgE-containing cells (before 24 ± 6 , after 6 ± 2 , $p < 0.001$) and a significant increase in IgA cell numbers (before 81 ± 9 , after 124 ± 7 , $p < 0.001$). IgM and IgD cell numbers fell but did not reach statistical significance (Student's t test for paired data). There was no change in IgG cell numbers. The changes in IgA and IgE cell numbers in individual patients are illustrated in Fig. 5.1.

CONCLUSIONS

In the present study a significant difference in the numbers of cells containing IgA or IgM from controls could not be demonstrated, although differences tending to support the findings of Popovic et al., (1974) were noted, i.e. that at the time of infection IgA cell numbers fall and IgM cell numbers increase. Following successful treatment of giardiasis the numbers of IgA-containing cells rose significantly. A previously unreported rise in the number of IgE and IgD-containing cells was also

observed. The response to treatment suggests that these latter changes are transient and result from the infection. These data do not support the concept based on the previous demonstration of decreased secretory IgA levels that symptomatic infections in man are the result of a pre-existing deficiency of IgA in intestinal secretions (Zinneman and Kaplan, 1972), since IgA synthesis seemed to increase later in the course of the infection. It is possible that mucosal IgA production is inhibited in some way by the parasite, but this possibility would require further study.

2. INTRAEPITHELIAL LYMPHOCYTE COUNTS IN PATIENTS WITH GIARDIASIS

The data in this section are derived from two sources:

1. Using the histological specimens obtained from the patients described in Chapter 3 as presenting to a gastro-intestinal unit in the U.K., intraepithelial lymphocyte counts were performed before and after successful treatment of giardiasis with metronidazole.
2. In France I was able to obtain jejunal biopsies of patients with giardiasis from the Department of Pathology, mainly those of the patients used for the study of mucosal immunoglobulin-containing cells described in the first part of this chapter, for estimation of intraepithelial lymphocytes.

In both cases the counts were performed as described in Chapter 2, without knowledge of whether the biopsy under examination had been taken before or after treatment.

RESULTS

The results are shown in Fig. 5.2. Counts were performed before treatment on 20 patients, 8 in the U.K. and 12 in France. All but 4 fell within the normal range of 6 - 40 IE lymphocytes/100 epithelial cells, and the mean value of 32.3 ± 4.12 was clearly within the normal range. Of the 4 raised counts, 3 were only moderately raised (48, 48 and 51), while one patient had a very high count of 89 IE lymphocytes/100 epithelial cells (patient 1 in Chapter 4). The height of the IEL count appeared to correlate with the degree of histological abnormality, in that the 3 U.K. patients with raised counts all had some degree of villus atrophy and marked inflammatory cell infiltrates. The histological appearances of the French patient with a raised count are not known.

Biopsies were available from only 7 of these patients after treatment, in most cases because patients did not wish to submit to a second biopsy. All but one of these counts fell within the normal range, and the mean count of 31.1 ± 3.63 was almost identical to the pre-treatment mean. However, dramatic falls in the IE lymphocyte count were seen in the 3 out of the 4 patients with initially

raised counts in whom a post-treatment count was possible. Both patients with pre-treatment counts of 48 IEL/100 epithelial cells had post-treatment counts of 24 IEL/100 epithelial cells, while the patient with a pre-treatment IEL count of 89 had a post-treatment count of 47, in spite of complete reversion of the other histological abnormalities to normal. Three patients showed a modest rise in IEL count following treatment, but in each case the post-treatment value remained within the normal range.

CONCLUSIONS

These data demonstrate that the majority of patients with giardiasis have normal IE lymphocyte counts. Raised counts were seen in those patients with histological evidence of villus atrophy, and in these patients the IEL count returned towards normal in parallel with the histological appearances following treatment.

Only two previous studies of IEL counts in patients with giardiasis have been reported. Wright and Tomkins (1977) found that in adults with giardiasis the IEL count tended to correlate with the degree of functional and histological abnormality, and that initially raised counts tended to fall with treatment. Ferguson et al., (1976) reported raised counts in children with giardiasis.

It has been suggested that villus atrophy is likely to be an expression of tissue damage resulting from T-cell

mediated immune responses in the small intestine (Ferguson and Jarrett 1975, McDonald and Ferguson 1977). Most IE lymphocytes are T cells (Ferguson 1977). The correlation between the IEL count and the degree of histological abnormality is therefore unsurprising, and provides evidence for the importance of cell-mediated immunity in giardiasis.

TABLE 5.1

TOTAL NUMBERS \pm 1 SEM OF LAMINA PROPRIA Ig-CONTAINING CELLS OF EACH IMMUNOGLOBULIN CLASS, EXPRESSED AS BOTH TOTAL NUMBERS PER 3 TISSUE UNITS AND AS PERCENTAGE OF THE

TOTAL IMMUNOFLUORESCENT CELL NUMBERS, IN 20 PATIENTS WITH UNTREATED GIARDIASIS AND 25 CONTROLS.

| | TOTAL NUMBER OF L.P. Ig-CONTAINING CELLS | | | | |
|---------------------------------------|---|---------------------|--------------------|---------------------|---------------------|
| | IgA | IgM | IgG | IgD | IgE |
| Controls n = 25 | 140 \pm 14.2 100% | 19 \pm 3 14.0% | 14 \pm 3 9.5% | 2 \pm 0.5 1.5% | 2 \pm 0.5 1.5% |
| Giardiasis patients n = 20 | 156 \pm 21 100% | 33 \pm 8 20% | 15 \pm 10 9% | 7 \pm 2 4% | 22 \pm 5 13% |
| Student's t test for unpaired data | N.S. | N.S. | N.S. | p < 0.005 | p < 0.001 |

L.P. = Lamina propria

TABLE 5.2

TOTAL NUMBERS \pm 1 SEM OF LAMINA PROPRIA Ig-CONTAINING CELLS OF EACH IMMUNOGLOBULIN CLASS, EXPRESSED AS IN TABLE 5.1, IN 10 PATIENTS BEFORE AND AFTER TREATMENT OF GIARDIASIS.

| TOTAL NUMBER OF L.P. Ig-CONTAINING CELLS | | | | | | |
|---|------------------|----------------|---------------|----------------|-------------|---------------|
| | | <u>IgA</u> | <u>IgM</u> | <u>IgG</u> | <u>IgD</u> | <u>IgE</u> |
| Before treatment n = 10 | 153 ± 51 100% | 81 ± 9 53% | 26 ± 3 17% | 15 ± 13 10% | 7 ± 2 5% | 24 ± 6 15% |
| After treatment n = 10 | 170 ± 18 100% | 124 ± 7 73% | 20 ± 5 12% | 15 ± 5 9% | 5 ± 2 3% | 6 ± 2 3% |
| Student's t test for paired data | NS | P < 0.001 | NS | NS | NS | p < 0.01 |

L.P. = Lamina Propria

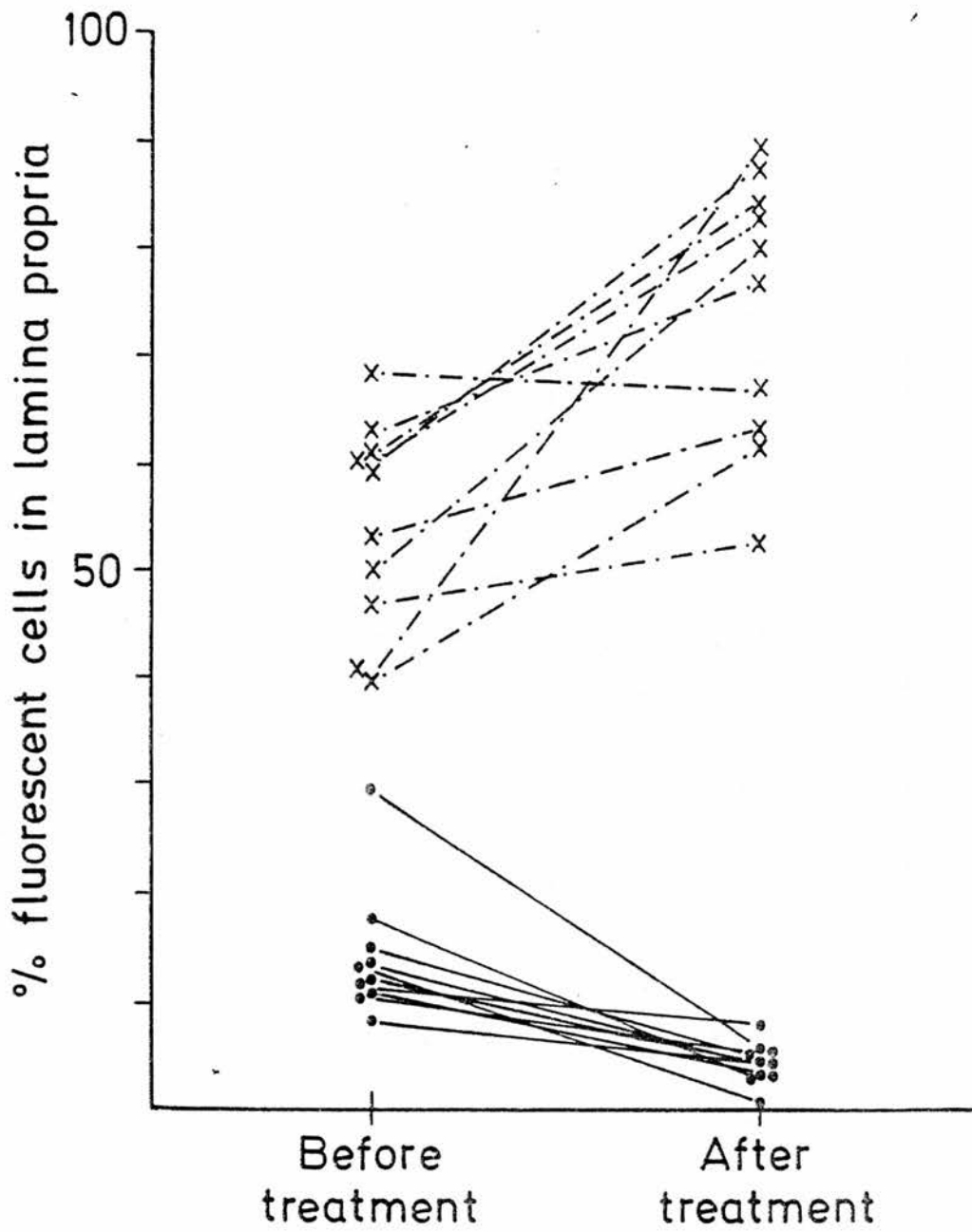


Figure 5.1

Lamina propria IgA and IgE cell numbers in patients with giardiasis before and after treatment. (X — — — X; • — — — •).

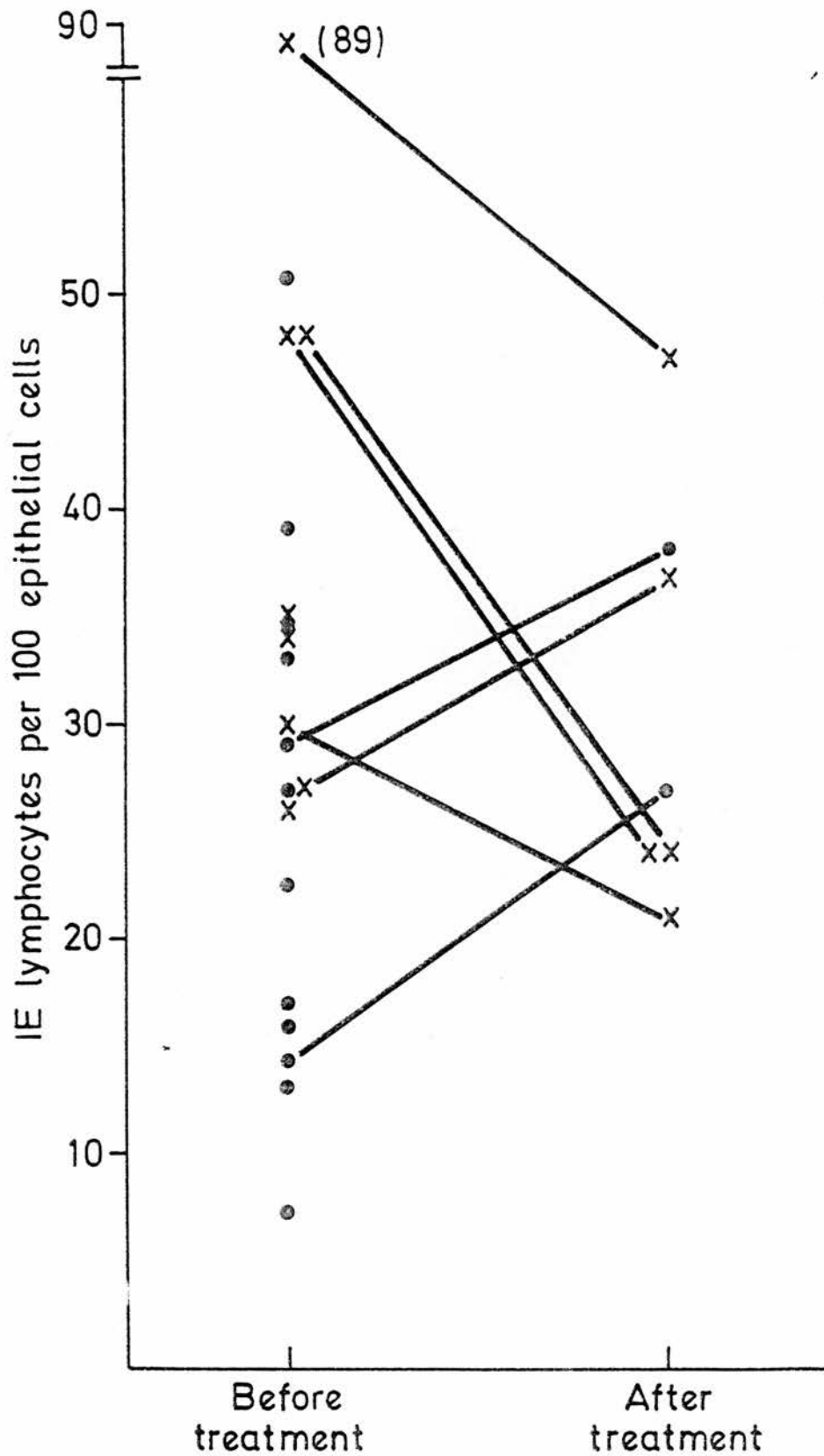


Figure 5.2

IE lymphocyte counts in giardiasis patients before and after treatment (● - French patients X - UK patients).

CHAPTER 6

THE DEVELOPMENT OF ACCURATE METHODS OF
MEASURING TROPHOZOITE NUMBERS IN THE SMALL INTESTINE
OF MICE INFECTED WITH GIARDIA MURIS AND THE EFFECTS OF
IMMUNOSUPPRESSION ON PRIMARY AND LATENT INFECTION

INTRODUCTION

In their original description of the mouse model of giardiasis, Roberts-Thomson and colleagues (1976(a)) assessed the magnitude and duration of infection by two methods. To measure faecal cyst excretion they isolated mice in individual cages for 2 hours, collected the resulting faeces and concentrated and washed the cysts by a centrifugation method. The trophozoite load was measured by flushing saline through the whole small intestine and counting after centrifugation. Although these methods appeared to correlate in demonstrating the peak of infection, cyst counts are notoriously unreliable, and from time to time in our laboratory we have found that encystation did not occur reliably, necessitating the transfer of trophozoites to ensure the continuation of the model. When we attempted the method of trophozoite counting described by Roberts-Thomson et al., however, it became clear on histological examination of the mucosa after the saline wash-through that many trophozoites remained attached to the epithelium.

I therefore set out to develop an accurate method of enumerating the trophozoites in the small intestine. The first method described in this chapter proved unsatisfactory for measuring total trophozoite numbers, but was useful in demonstrating the distribution of parasites within the small bowel. The final counting method improved the return of trophozoites by a factor of 10 when compared with Roberts-Thomson's, and also led to the discovery that

after self-cure the infection may become latent. These phenomena were investigated further by treating infected mice with immunosuppressive agents.

1. THE DEVELOPMENT OF ACCURATE METHODS OF COUNTING TROPHOZOITES

Trophozoite counts - sieve method

Since the trophozoites adhere closely to the mucosa the initial aim was to devise a method which did not depend on dislodging the parasite from the epithelium. The whole small intestine was removed and its length measured. Beginning at the gastro-duodenal junction, segments of whole intestine 1 cm in length were taken, leaving 2 cm between each one. Thus counts were performed on 1/3 of the small intestine and the results extrapolated to obtain a total count for the whole small intestine. Each 1 cm segment was mashed through a stainless steel grid with holes of 50 μ diameter, using a glass rod and washing through with 1 ml normal saline from a syringe and needle. Aliquots of the resulting suspension were then examined and a trophozoite count performed in an improved Neubauer haemocytometer.

The results of trophozoite counts using this method in following the course of infection are shown in Fig. 6.1. The peak trophozoite count occurred at day 23 and amounted to 6.7×10^6 trophozoites/small intestine. This compared

with peak trophozoite counts of the order of 2.0×10^6 in the original description. The infection is seen to take longer to resolve than had been demonstrated by the saline wash technique, and trophozoites were still detectable in the animal studied at 2 months after inoculation.

This method is also useful in showing the distribution of parasites along the small intestine at different stages of the infection (Fig. 6.2). These results confirm previous observations that the greatest numbers of trophozoites are to be found in the proximal small intestine, although early in the course of infection large numbers may be found in the ileum. As the infection progresses the localisation becomes more obviously confined to the upper jejunum.

The main disadvantage of this method is that it is laborious and time-consuming, and some trophozoites may be damaged in the process. Also, the counts are technically difficult because of the amount of cellular debris. The following method of dislodging trophozoites from the mucosa without trauma was therefore developed.

Trophozoite counts - vibration method (Gaginella et al., 1978)

Mechanical vibration of everted intestine in a suitable medium is a standard method of obtaining enterocytes for experimental purposes. When this method was applied to the intestine of Giardia infected mice it was found that the trophozoites were dislodged into the medium in large numbers. The medium used consisted of 98 ml normal saline

and 2 ml acetyl cysteine 20% ('Airbron') as a mucolytic agent. After killing the animal the small intestine is removed intact, and the mesentery peeled off. Beginning at the duodenum, the gut is then everted onto a spiral rod of glass or steel, exposing the luminal surface. The ends are tied with silk sutures. The intestine is then vibrated in 100 ml medium at full power for 10 minutes using a Chemap AG Vibromixer Model Type E1 (Fig. 6.3). After agitating the suspension thus obtained a 10 ml sample is removed and spun at 430 G for 10 minutes. 9.5 ml supernatant is removed and the pellet resuspended in the remaining 0.5 ml medium. The number of trophozoites is then counted in the haemocytometer, and the number present in the whole small intestine is calculated.

A time period for vibration of 10 minutes was chosen because studies in 5 animals, in which the fluid medium was changed at 10 minute intervals showed that between 97 and 100% of recovered trophozoites were present in the 10 minute vibration sample (Table 6.1). The limit of detection of trophozoites with this method is 1×10^4 trophozoites/whole small intestine. The absence of Giardia in the intestine after vibration was confirmed histologically and by examination of mucosal smears taken after vibration.

Cyst and trophozoite counts in Giardia muris infection of CBA mice

Cyst excretion after infection with 1,000 cysts was examined in 8 CBA mice at 1, 2, 3, 4 and 6 weeks post-infection.

In addition, cyst counts were carried out in 50 CBA mice which had been infected between 3 and 11 months previously, for standard maintenance of the infection.

Thirty-seven infected mice were killed at intervals up to 6 weeks post-infection (6 - 9 animals per group) and in each animal the trophozoites were counted by the vibration technique. A further 18 stock CBA mice, 3 - 11 months post-infection, were killed and trophozoites counted by the vibration technique.

The results are shown in Fig. 6.4. They confirm that the results of 2 hour cyst counts show a pattern of cyst excretion very similar to previous reports (Roberts-Thomson et al., 1976) with the peak cyst excretion at 2 weeks, and cysts being undetectable in the majority of animals after 4 weeks. The number of trophozoites also reached a peak at 2 weeks post-infection, with values in some of the animals as high as $15 - 18 \times 10^6$. This return is approximately double that which was obtained by the sieve technique and almost tenfold higher than reported trophozoite counts using the saline wash method (Roberts-Thomson et al., 1976). Furthermore, trophozoites were found in considerable numbers at 6 weeks post-infection when cysts were not detected in the faeces, and were also present in 50% of the animals studied at 3 or more months post-infection.

2. DOES HOST RESISTANCE TO SECONDARY INFECTION
DEPEND ON LATENT INFECTION
WITH SMALL NUMBERS OF PARASITES?

The discovery, using the new trophozoite counting methods described above, that small numbers of parasites may remain in the small intestine for several months after a primary infection led me to wonder about the nature of the relationship between the host and the small number of parasites remaining. It had previously been demonstrated both in human volunteers (Rendtorff 1954) and in mice (Roberts-Thomson et al., 1976) that a primary infection confers resistance to subsequent challenges with the organism. It had been assumed that this resistance was due to protective immunity in the host, but our data raised the possibility that the host's resistance might in some way be dependent on the presence of the parasite in small numbers, perhaps as a persistent antigenic stimulus. I therefore treated a group of 18 BALB/c mice with oral metronidazole 366 mg/Kg for 11 days by gavage beginning on day 28 after a primary infection. This dose of metronidazole has been shown to be effective in eradicating murine giardiasis (Mandoul et al., 1961) and 2 treated mice killed on day 52 had no detectable trophozoites. Four weeks later these mice, and a control group of the same age and sex, infected at the same time but not having been treated with metronidazole were challenged with 1,000 cysts orally per mouse.

RESULTS

The magnitude of primary infection was similar in both groups as judged by cyst and trophozoite counts, although slightly lower trophozoite counts were noted in the group designated for metronidazole treatment. Faecal cyst counts became negative in both groups by day 28, and remained so in both groups at days 7 and 14 after reinoculation. Trophozoite counts performed 7 and 14 days after reinoculation confirmed that the metronidazole-treated mice were resistant to reinfection, although 5 of the 12 had small numbers of trophozoites present (Fig. 6.5). At day 28 after reinfection 5 of 6 mice tested had no detectable trophozoites. At days 7 and 14 after reinoculation trophozoites were found in most of the control mice in slightly greater numbers, probably indicating persistence of the primary infection rather than any increase in susceptibility to reinfection as compared with the metronidazole-treated mice.

Thus the small numbers of trophozoites persisting after cyst counts had become negative did not appear to be important in the maintenance of resistance to reinfection. Could they perhaps lead to recurrence of infection if the host's immunity was in some way impaired? This question led us to examine the effects of immunosuppression not only on the latent infection which we had discovered, but also on the primary infection itself.

3. EFFECTS OF IMMUNOSUPPRESSION ON PRIMARY AND LATENT MURINE GIARDIASIS

While experiments which had been designed to examine the effects of corticosteroid treatment were under way in Edinburgh (described below), I attempted to treat mice with 2 different forms of immunosuppressive agent prior to primary infection in France. One group of 10 BALB/c mice was given the mast cell stabilising agent disodium cromoglycate in their water supply in a concentration of 40 mg/50 mls water. This was calculated to give approximately 60 mg/Kg/day, and was continued for 2 days prior to infection, and for the entire 28 day period after infection. Several other groups of mice received the organochlorine pesticide Lindane, incorporated into otherwise normal food pellets, in a concentration of 150 ppm for varying periods prior to and in some cases during primary infection with Giardia muris. This compound has been shown to have a variety of potentially important immunosuppressive properties, including effects on IgE-mediated reactions (André et al., 1981), and preliminary results using small numbers of mice had shown that pretreatment with this substance for at least 10 days may prolong the infection.

Unfortunately these experiments, involving large numbers of mice and taking several months to complete, were rendered invalid by virtue of the fact that most of the mice coincidentally became heavily infected with Hexamita muris, a common murine protozoal parasite. This led to a

poor 'take' of infection with Giardia, very low cyst counts at the peak of infection in both treated and control mice, and very small numbers of trophozoites in all groups when sacrificed at 4 weeks after initial challenge. No conclusions could therefore be drawn and time did not permit me to repeat this work.

EFFECT OF CORTISONE ACETATE PRETREATMENT ON
TROPHOZOITE NUMBERS IN A
PRIMARY GIARDIA MURIS INFECTION

This experiment was designed to establish the effect of cortisone acetate, given one day prior to oral inoculation of cysts, on the time-course of primary infection and on the magnitude of the parasite load. Thirty-five male CBA mice were injected with 2.5 mg cortisone acetate subcutaneously in the interscapular region and a further 35, matched for age and sex, had 1 ml of saline subcutaneously. The following day all animals were infected with 1,000 Giardia muris cysts. Five animals from each group were killed at 1, 2, 3, 4, 6, 8 and 10 weeks post-infection. Trophozoite counts were carried out using the vibration method as described in previous chapters. Results, shown in Figure 6.6 illustrate that in this experiment, trophozoite counts of saline treated animals reached a plateau between one and six weeks post-infection, with values of between 5,000,000 and 10,000,000 trophozoites per animal.

In contrast, the trophozoite counts in cortisone pretreated animals were significantly higher than controls at 1, 2, 3 and 4 weeks post-infection, values for individual animals at weeks 2 and 3 reaching as high as 25,000,000 trophozoites. In both groups, there was a drop in trophozoite counts at 8 and 10 weeks post-infection, and for the group of cortisone pretreated animals, the mean count at 8 weeks was significantly higher than in the saline pretreated controls.

Thus, this experiment showed that cortisone acetate pretreatment modified the parasite load in primary Giardia infection, with substantially higher parasite numbers early in the course of infection (three-fold increase at 2 weeks) and with a slower rate of drop in parasite numbers at 2 months post-infection.

In order to differentiate an effect of cortisone on the parasite, from an effect on the specific and non-specific host immunity, animals were given cortisone acetate one and two weeks prior to Giardia infection and trophozoite counts were done in both groups of cortisone pretreated animals, and in saline injected controls, on the tenth day post-infection. Results, illustrated in Figure 6.7 show that the parasite load was significantly higher than control in both cortisone acetate treated groups of animals. It is therefore likely that the effect of cortisone is exerted on the host, rather than by direct effect on the parasite.

Recrudescence of chronic Giardia infection

This aspect was studied in a group of 31 animals which had been infected with Giardia muris 8 months previously. Nine of the animals were killed and trophozoite counts were done. Trophozoites were detected in only 2 of these 9 mice. The remaining 22 animals were given 2.5 mg of cortisone acetate subcutaneously, and were killed in groups at 1, 2, 3, 5 and 10 days after cortisone injection. Trophozoite counts were carried out. Results of this experiment are shown in Figure 6.8. A few trophozoites were detected in one of the four animals killed on day one post-cortisone; thereafter, values of the order of 5 to 25,000,000 trophozoites were obtained for 10 of the animals and 2 others had lower trophozoite counts. Trophozoites were not detected in 5.

It seems likely that 25% of animals have completely cleared the parasite by 8 months after primary infection. A further 50% have small numbers of trophozoites, below the limit of detection of this assay system which was 10,000 trophozoites per animal; but after a single injection of cortisone acetate the trophozoite numbers increase very rapidly, to reach values analogous to those in a primary infection, by the second day after cortisone administration.

CONCLUSIONS

Two different methods were developed for the isolation of live Giardia trophozoites from the intestines of infected mice. The first (sieve) method gave a yield of trophozoites approximately 5 times that described in previous reports (Roberts-Thomson et al., (1976a)); and proved most useful in defining the distribution of the trophozoites in the intestine. The second (vibration) method proved to be the method of choice for obtaining accurate measurements of trophozoite numbers, a 10 minute period of vibration being sufficient to extract 97 - 100% of the trophozoites present, the figures obtained indicating that the method of Roberts-Thomson et al., underestimated the trophozoite load by a factor of approximately 10. The two methods are thus seen to be complementary. In addition to this the vibration method revealed that trophozoites persist for at least 3 months in the small intestine of 50% of mice which had apparently undergone self-cure of their primary infection. This phenomenon has not previously been reported.

The experiments described have shown that the small numbers of parasites remaining in the intestine after apparent self-cure of a primary infection probably do not contribute to the host's resistance to subsequent infection. In such mice a single injection of cortisone acetate induced recrudescence of Giardia infection within 48 hours, and this confirmed that approximately 75% of the animals studied still had a few parasites within the intestine even 8 months after

infection. Cortisone acetate pretreatment also had significant effects on the course of a primary infection, increasing the number of parasites in the intestine at the peak of the infection and also prolonging its course. It therefore seems likely that the numbers of parasites are limited at all stages of the infection by the actions of the host immune system. These observations raise many questions and suggest several possible avenues for future research. This is discussed further in Chapter 10.

TABLE 6.1

| <u>TROPHOZOITE COUNT</u> | | |
|--------------------------|-------------------|-------------------|
| <u>ANIMAL</u> | <u>PERIOD 1</u> | <u>PERIOD 2</u> |
| 1 | 9.1×10^6 | 5×10^4 |
| 2 | 7.8×10^6 | 5×10^4 |
| 3 | 3.4×10^6 | 5×10^4 |
| 4 | 8×10^6 | 1.5×10^5 |
| 5 | 13×10^6 | 1.5×10^5 |

Results of experiment to verify that vibration for 10 minutes provides optimal return of trophozoites. Animals were male CBA mice infected 10 - 14 days previously. The everted intestine was vibrated for successive periods of 10 minutes.

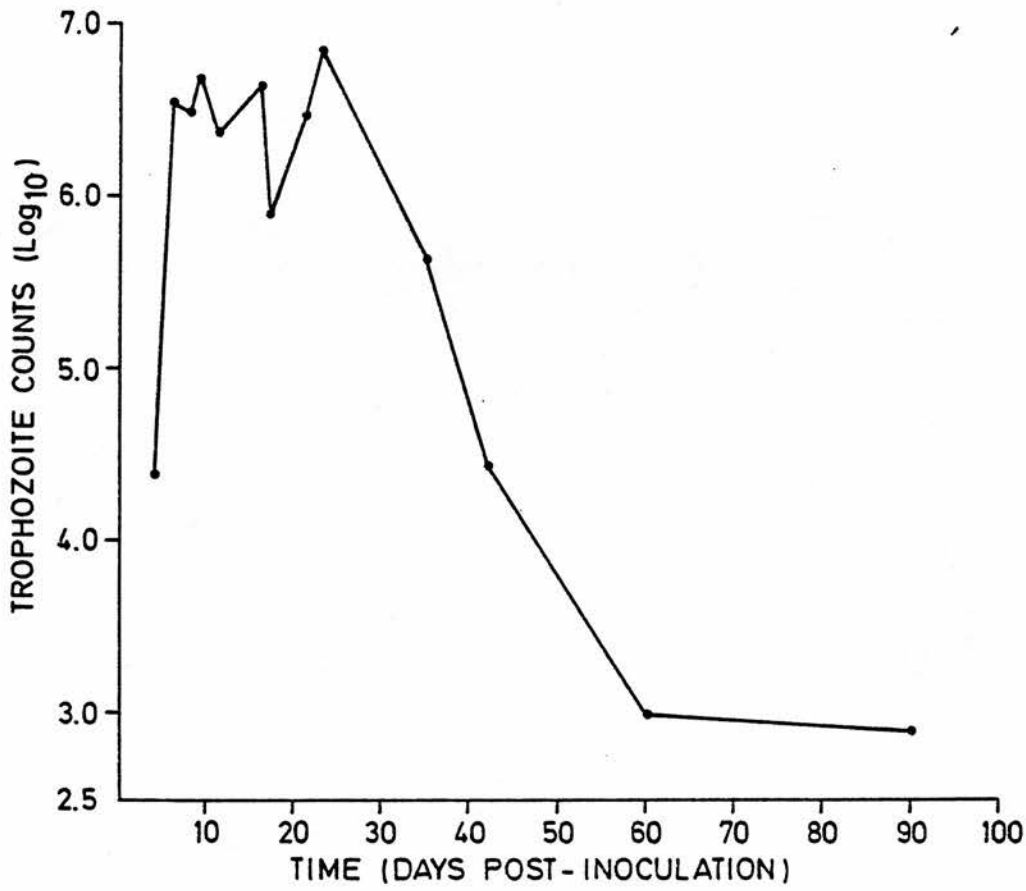


Figure 6.1 Trophozoite counts in primary
Giardia muris infection of CBA mice -
sieve method.

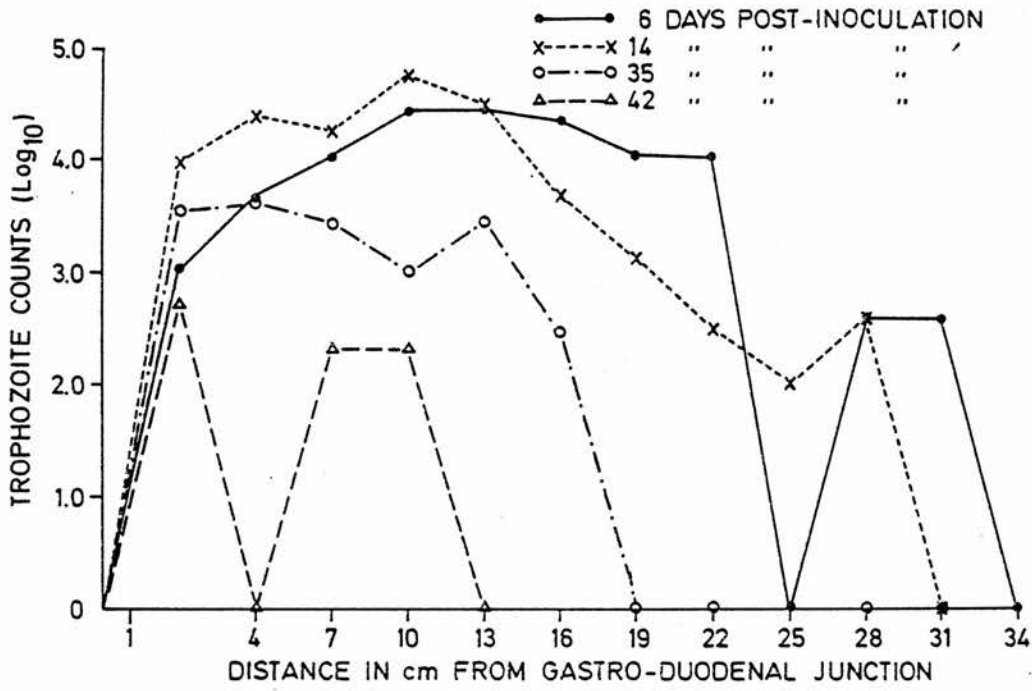


Figure 6.2

Distribution of trophozoites in the small intestine of CBA mice infected with *Giardia muris*, and variation with time after inoculation.

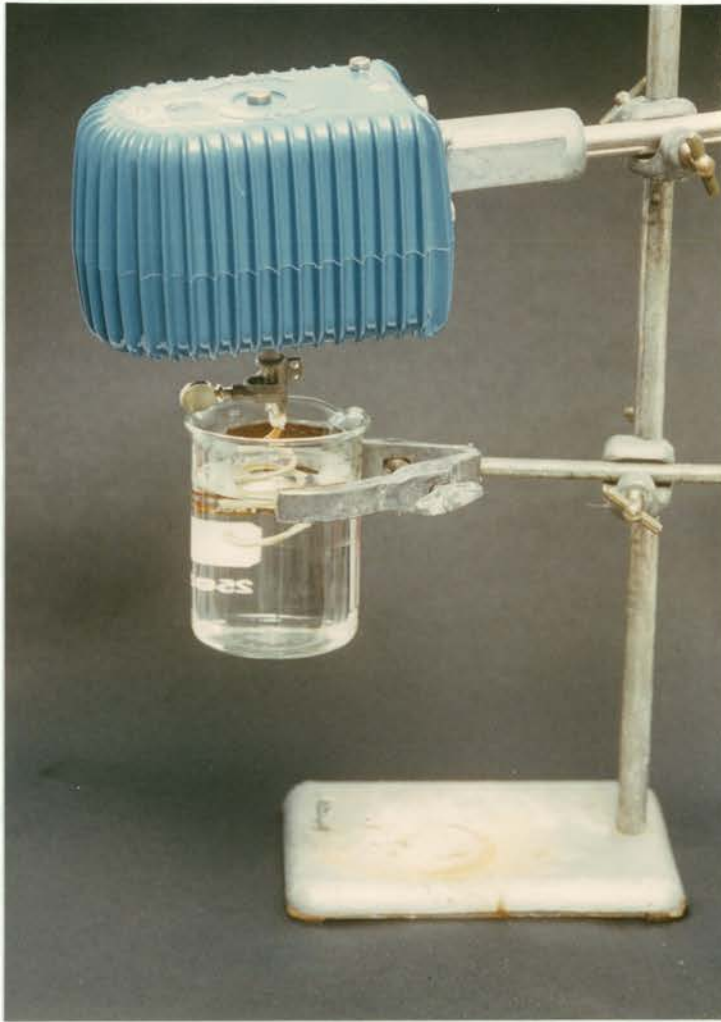


Figure 6.3 Isolation of *Giardia muris* trophozoites from the everted small intestine of infected mice - the vibromixer with attached glass spiral, on which the intestine is secured.

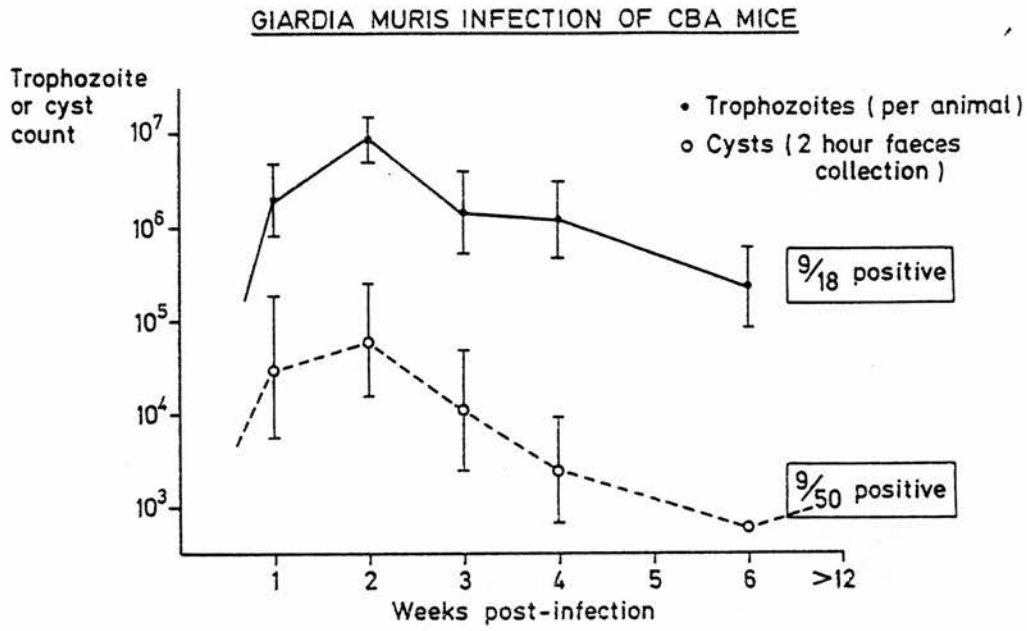


Figure 6.4 Comparison of cyst and trophozoite counting methods during the course of a primary Giardia muris infection in CBA mice.

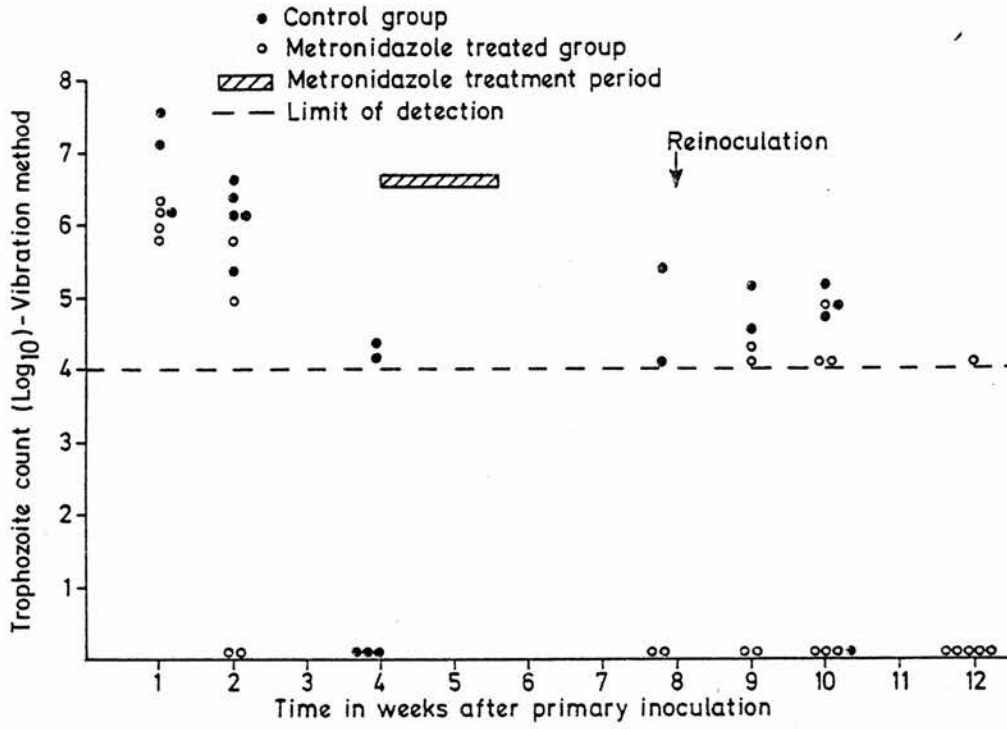


Figure 6.5

The effect of treatment of primary *Giardia muris* infection with metronidazole on the response to subsequent oral inoculation with cysts.

GIARDIA MURIS INFECTION OF CBA MICE

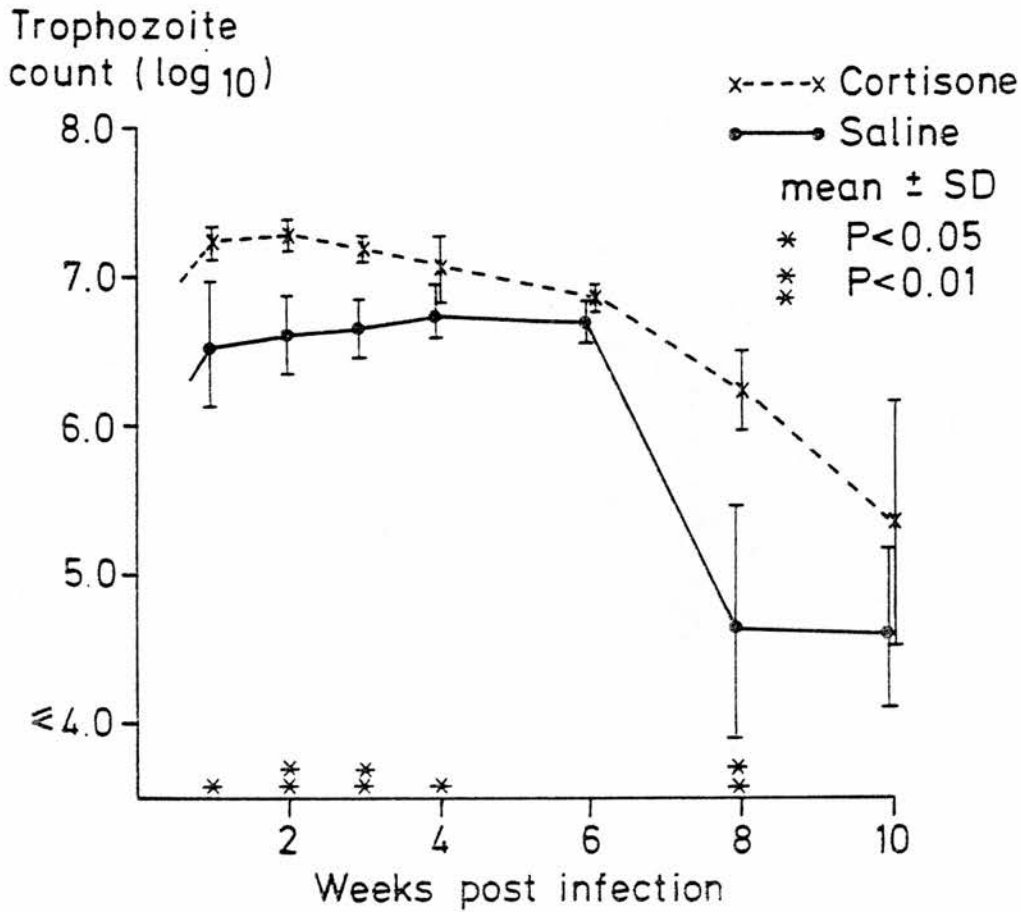


Figure 6.6 Effect of pre-treatment with cortisone acetate on trophozoite numbers during the course of a primary Giardia muris infection.

GIARDIA MURIS INFECTION OF CBA MICE

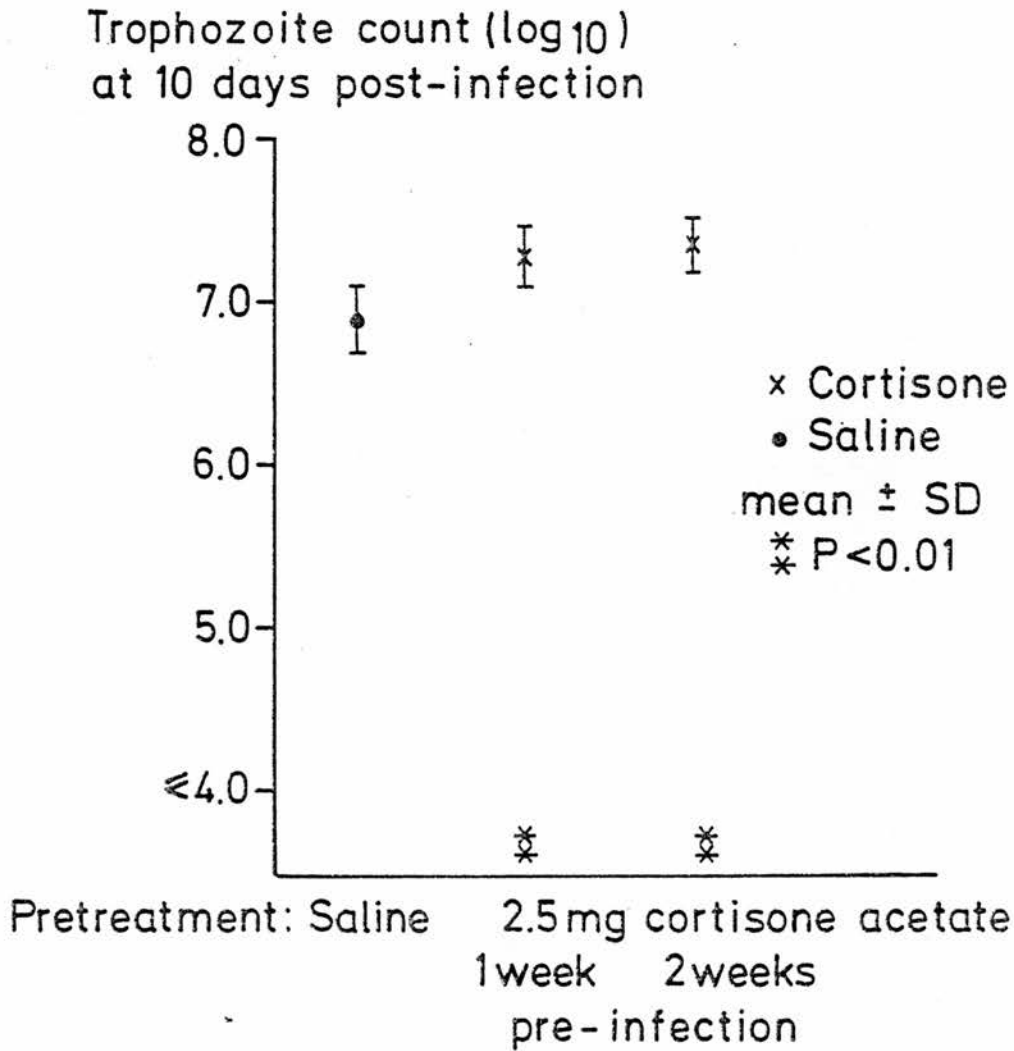


Figure 6.7 Effect of pre-treatment with cortisone acetate at 1 and 2 weeks prior to inoculation on trophozoite counts at 10 days post-inoculation.

CBA MICE, 8 MONTHS AFTER GIARDIA MURIS INFECTION

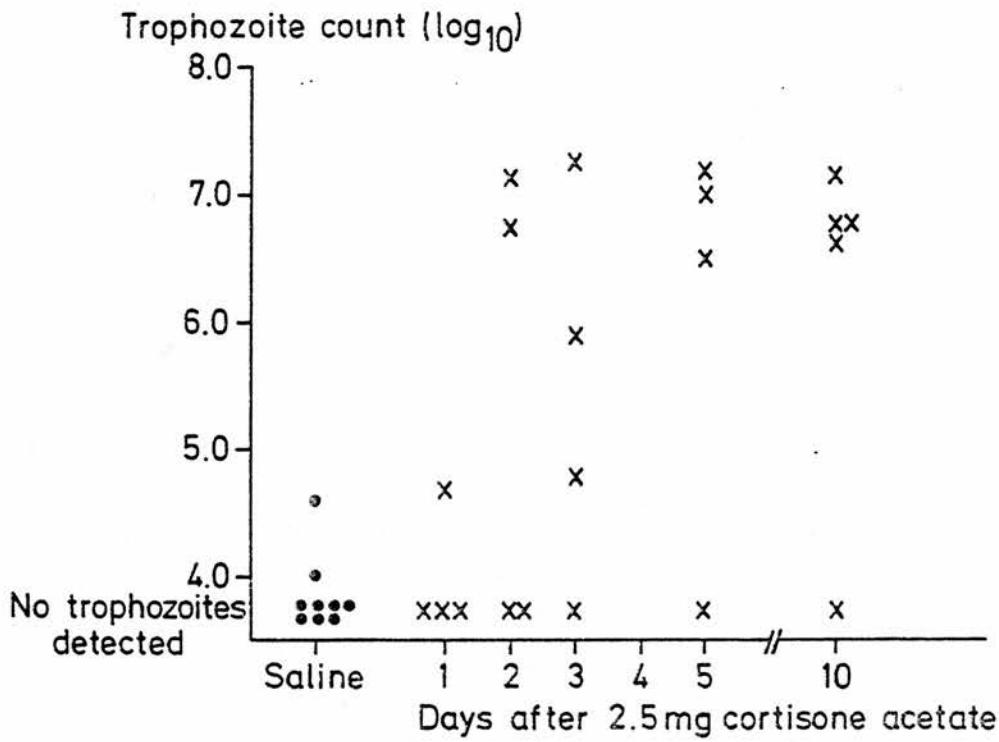


Figure 6.8 Effect of cortisone acetate treatment on trophozoite counts in CBA mice 8 months after primary infection with Giardia muris.

CHAPTER 7

PATHOLOGICAL CHANGES IN THE SMALL INTESTINE OF MICE
EXPERIMENTALLY INFECTED WITH GIARDIA MURIS

INTRODUCTION

The experiments described in this chapter were designed to examine the progression of changes in mucosal architecture, epithelial cell kinetics and brush border enzymes during a primary Giardia muris infection. The objectives were to establish which, if any, of the pathological features appear to be correlated with the magnitude of the parasite load at that site, and whether any of these features are likely to be due to mechanisms other than the direct effect of the parasite.

EXPERIMENTAL METHODS

For these experiments batches of 10 - 15 mice were infected with 1,000 Giardia muris cysts orally; groups of animals were then killed at 1, 2, 3, 4, 6, 8 and 10 weeks post-infection. One group of uninfected (control) mice was also studied. Each mouse was given intraperitoneal colchicine and killed at a measured time interval thereafter, and specimens of jejunum and ileum taken for microdissection, and for histology as described in Chapter 2.

RESULTS

In Table 7.1 are summarised the results of studies in histological sections of jejunum and ileum, over the 10 weeks after primary infection of CBA mice with 1,000 Giardia muris cysts. It is clear that, just as was shown by the quantitative assay (Chapter 6) the highest numbers of trophozoites are present in the jejunum in the first 4 weeks post-infection with few or no parasites visible at 8 and 10 weeks. In the ileum, confluent sheets of trophozoites were seen in only 4 of the 13 mice examined at one week post-infection, and few trophozoites were present in the majority of animals at one to four weeks post-infection.

Effects of primary Giardia muris infection on the lengths of villi and crypts

Results of the measurements of crypts and villi in jejunum and ileum are summarised in Figs 7.1 and 7.2.

In the jejunum significant shortening of villi was seen only at one week post-infection ($p < 0.01$), mean villus length being reduced from 690 μm to 630 μm . Villi were significantly longer ($p < 0.05$) than controls at 4, 6, 8 and 10 weeks post-infection. Significant crypt lengthening was also found between one and four weeks post-infection.

Results for the ileum showed a different pattern. Information is only available for 5 groups of animals (due to deterioration of other specimens in storage) and show

significant shortening of both villi and crypts at 8 and 10 weeks post-infection.

The effect of primary *Giardia muris* infection on crypt cell production rate (CCPR) in jejunum and ileum

Results of the measurement of epithelial cell kinetics are summarised in Table 7.2. Metaphase accumulation was linear in all groups over the period studied, and the jejunal CCPR for the batch of control animals was 6.2 mitoses/crypt/hour. Values were higher in jejunum of mice at 1, 2, 3, 4 and 8 weeks post-infection, but the only result which approached statistical significance was that at 3 weeks with p value between 0.1 and 0.05.

The crypt cell production rate was higher in the ileum than in the jejunum of control animals, being 8.6 mitoses/crypt/hour. In the ileum significantly lower values were present in the batches of mice killed at 3, 8 and 10 weeks post-infection.

Effect of primary *Giardia muris* infection on disaccharidase content of jejunum and ileum

Assays of tissue disaccharidases were carried out in a different batch of mice from those used in the epithelial cell kinetic study. There were 9 uninfected animals, and groups of 4 mice which had been infected with 1,000 *Giardia muris* cysts 1, 2, 3, 4, 6, 8 and 10 weeks previously. In contrast to the minimal effect of the infection on intestinal architecture, striking and significant disaccharidase changes were obtained.

At 2 weeks post-infection, lactase, sucrase and maltase levels in the jejunum were significantly lower than in control animals (Fig. 7.3). At 6, 8 and 10 weeks the disaccharidase content was higher than in the control mice. In the ileum the only abnormality in the early phase post-infection was a significant increase in lactase at one week and sucrase at 2 weeks post-infection (Fig. 7.4). However, significantly low values for lactase and sucrase were obtained in specimens taken at 6, 8 and 10 weeks post-infection.

SUMMARY OF THE ABNORMAL FEATURES AND CONCLUSIONS

In Table 7.3 the abnormalities of intestinal architecture and disaccharidases observed in these experiments are summarised. In jejunum, peak trophozoite numbers were present at 2 weeks with many parasites in the jejunum up to 4 weeks post-infection. A modest reduction in villus length occurred at week 1, and striking disaccharidase deficiencies at week 2. This was followed by lengthening of the crypts of Lieberkuhn and an elevated crypt cell production rate, peaking at week 3. Later after infection, as trophozoite numbers fell, there was an increase in both the length of the villi and the disaccharidase activity of the jejunum.

In contrast in the ileum few or no trophozoites were present during the course of this infection. The only

changes in the mucosa of the ileum in the early phase were an increase in the content of sucrase and lactase at weeks 1 and 2 respectively, and a decrease in crypt cell production rate at week 3. However, surprisingly, in specimens taken 8 and 10 weeks post-infection there was evidence of a reduction in the length of both crypts and villi, a reduced crypt cell production rate and reduced disaccharidase activity.

The changes seen in the ileum were the opposite of those in jejunum, suggesting rapid ileal adaptation. The results indicate that the disaccharidase deficiency associated with giardiasis is likely to represent a direct effect of the parasite on the brush border rather than enterocyte immaturity. Profound adaptive changes occur throughout the small intestine in response to a relatively localised insult.

TABLE 7.1

TROPHOZOITES (Tz) IN HISTOLOGICAL SECTIONS OF JEJUNUM AND ILEUM

IN CBA MICE INFECTED WITH 1,000 GIARDIA MURIS CYSTS

| EXPERIMENTAL GROUP (WEEKS POST- INFECTION) | JEJUNUM | | | | ILEUM | | | |
|---|---|------------------------------|-----------|------|---|------------------------------|-----------|------|
| | TOTAL NUMBER OF SPECIMENS EXAMINED | NUMBER OF SPECIMENS WITH: | | | TOTAL NUMBER OF SPECIMENS EXAMINED | NUMBER OF SPECIMENS WITH: | | |
| | | MANY Tz | FEW Tz | NONE | | MANY Tz | FEW Tz | NONE |
| 1 | 14 | 12 | 2 | - | 13 | 4 | 7 | 3 |
| 2 | 15 | 15 | - | - | 15 | - | 13 | 2 |
| 3 | 15 | 14 | 1 | - | 14 | - | 8 | 6 |
| 4 | 15 | 14 | - | 1 | 16 | - | 6 | 10 |
| 6 | 16 | 8 | 8 | - | 15 | - | - | 12 |
| 8 | 13 | 1 | 5 | 7 | 12 | - | - | 12 |
| 10 | 10 | 1 | 2 | 7 | 12 | - | - | 12 |

(Each specimen represents one animal)

TABLE 7.2

CRYPT CELL PRODUCTION RATE (CCPR) IN JEJUNUM AND ILEUM

OF CBA MICE INFECTED WITH 1,000 GIARDIA MURIS CYSTS

| EXPERIMENTAL GROUP (WEEKS POST- INFECTION) | CCPR - JEJUNUM | | CCPR - ILEUM | |
|---|--|--------------------------|--|--------------------------|
| | ACCUMULATED METAPHASES/ CRYPT/HOUR | P (VERSUS UNINFECTED) | ACCUMULATED METAPHASES/ CRYPT/HOUR | P (VERSUS UNINFECTED) |
| CONTROL | 6.2 | | 8.6 | |
| 1 | 7.1 | NS | - | |
| 2 | 7.0 | NS | - | |
| 3 | 8.2 | NS | 5.5 | NS |
| 4 | 7.5 | NS | - | |
| 6 | 5.9 | NS | 6.6 | NS |
| 8 | 7.0 | NS | 5.0 | P < 0.02 |
| 10 | 5.8 | NS | 5.3 | P < 0.05 |

TABLE 7.3
SUMMARY OF FEATURES OF SI PATHOLOGY
IN PRIMARY GIARDIA MURIS INFECTION OF CBA MICE

| | JEJUNUM | | | | | |
|-------------------------------|----------------------|--------------|------|------|------|------|
| | WEEKS POST-INFECTION | | | | | |
| | 1 | 2 | 3 | 4 | 6 | 8 10 |
| | Many | Max- imum | Many | Many | Few | Few |
| Trophozoites | ↑ | - | - | ↑ | ↑ | ↑ |
| Villus length | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| Crypt length | - | - | ↑ | - | - | - |
| Crypt cell production rate | - | - | ↑ | - | - | - |
| Disaccharidase | - | ↑ | - | - | ↑ | ↑ |
| | ILEUM | | | | | |
| | WEEKS POST-INFECTION | | | | | |
| | Few | Few | Few | Few | None | None |
| Trophozoites | - | - | - | - | - | - |
| Villus length | - | - | - | - | - | - |
| Crypt length | - | - | - | - | - | - |
| Crypt cell production rate | - | - | ↑ | - | - | - |
| Disaccharidase | - | ↑ | - | - | ↑ | ↑ |

GIARDIA MURIS INFECTION OF CBA MICE
INTESTINAL ARCHITECTURE - JEJUNUM

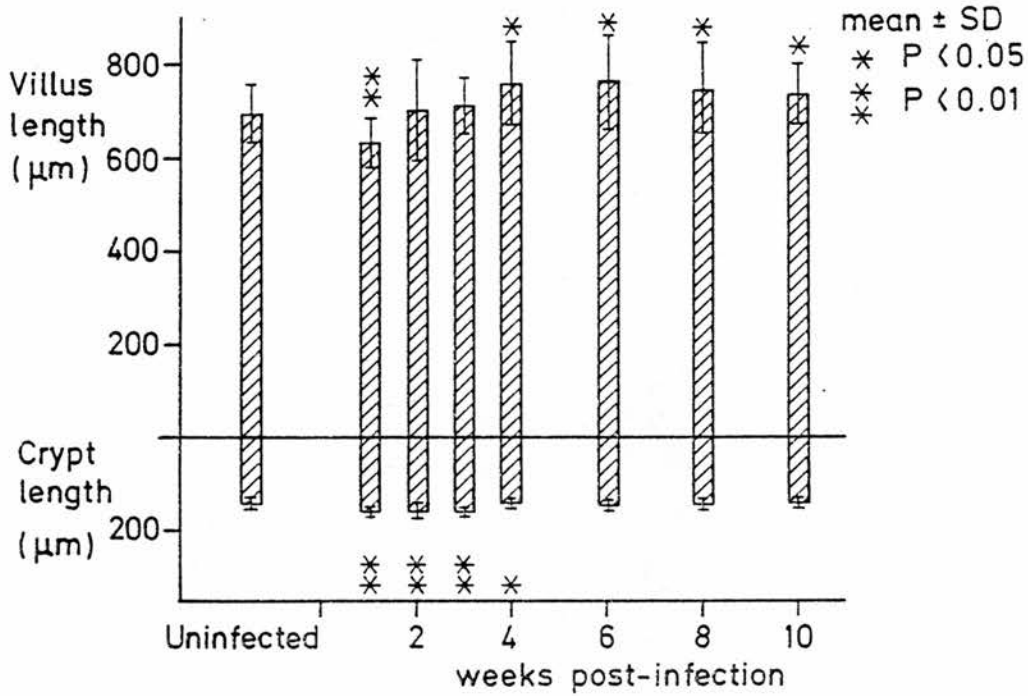


Figure 7.1 Diagrammatic representation of architecture of jejunal mucosa in groups of mice at intervals of one to 10 weeks after infection with Giardia muris. Ten to 15 mice per group (Mean \pm SD. * = $p < 0.05$, ** = $p < 0.01$, versus uninfected)

GIARDIA MURIS INFECTION OF CBA MICE
INTESTINAL ARCHITECTURE - ILEUM

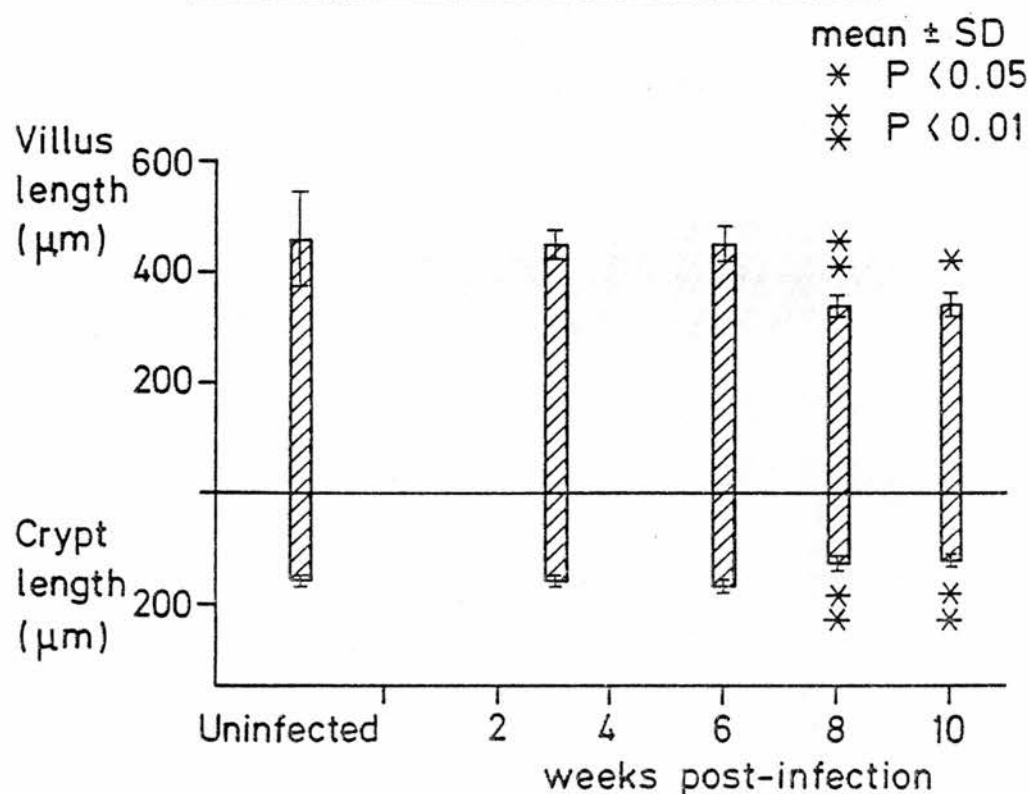


Figure 7.2

Diagrammatic representation of architecture of ileal mucosa in groups of mice at intervals of one to 10 weeks after infection with *Giardia muris*. Ten to 15 mice per group. (Mean \pm SD. * = $p < 0.05$), ** $p < 0.01$, versus uninfected).

GIARDIA MURIS INFECTION OF CBA MICE
DISACCHARIDASES - JEJUNUM

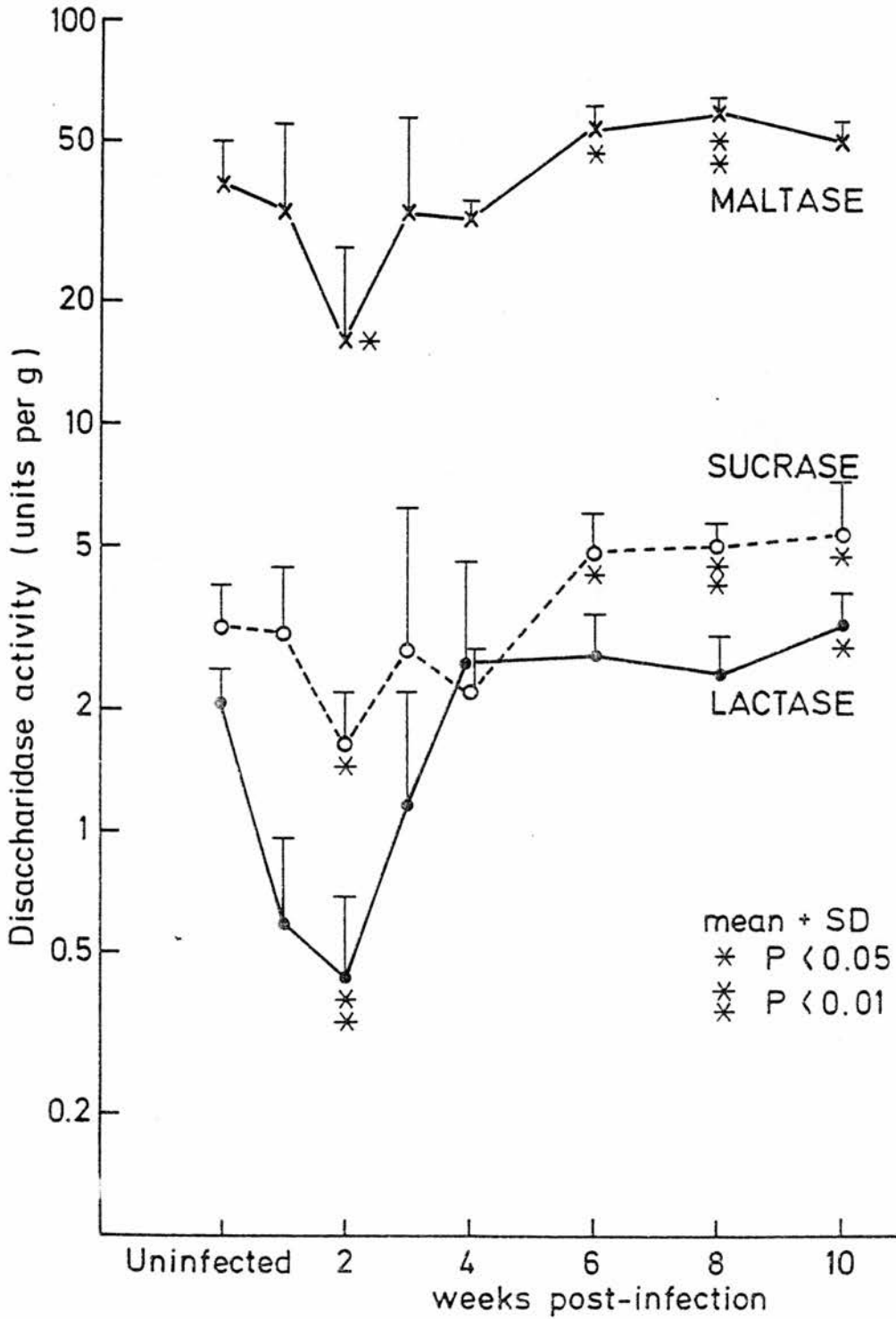


Figure 7.3

Effects of primary Giardia muris infection on disaccharidase content of jejunal mucosa in CBA mice at intervals of one to 10 weeks after inoculation with Giardia muris cysts.

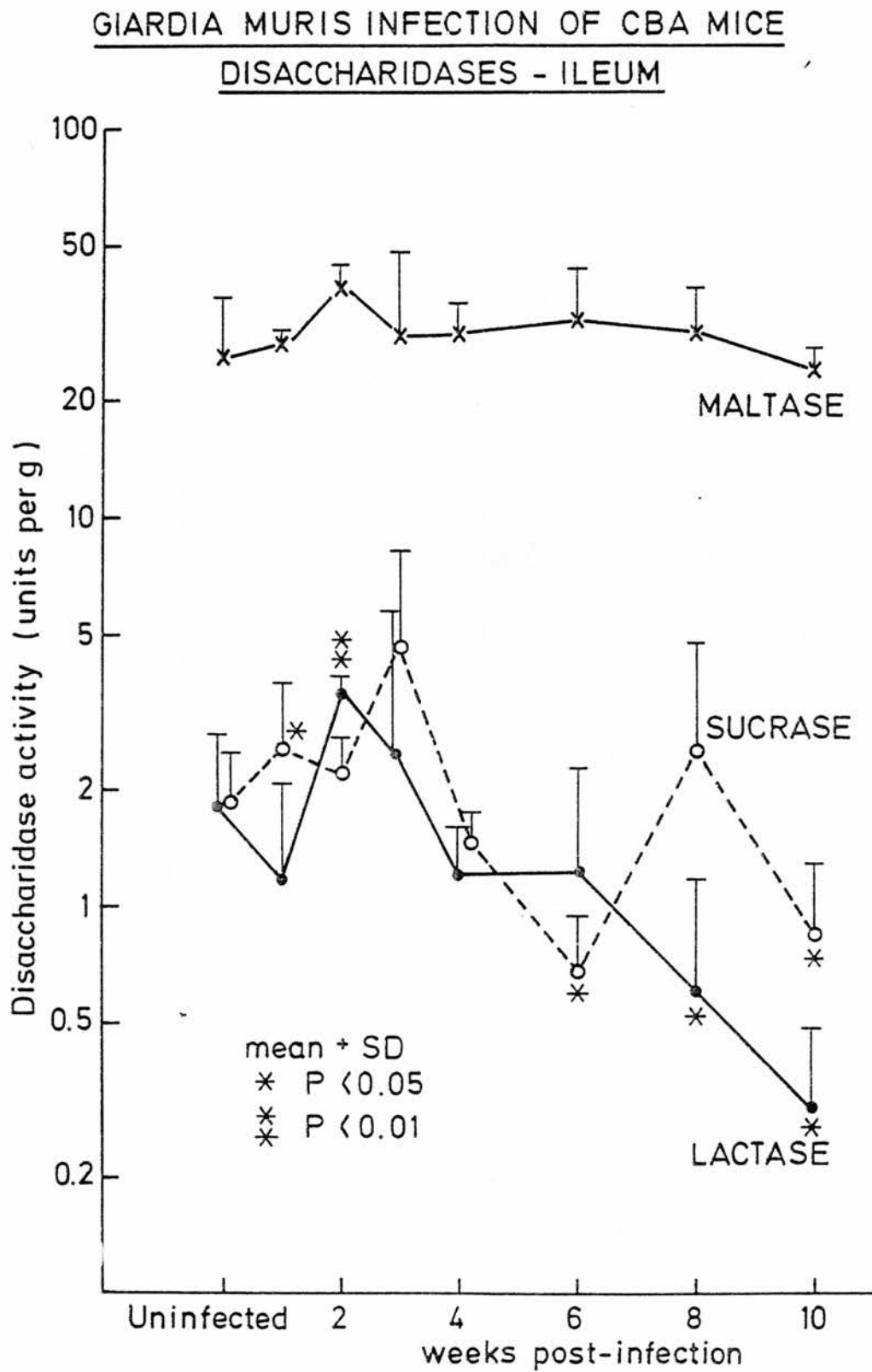


Figure 7.4

Effects of primary Giardia muris infection on disaccharidase content of ileal mucosa in CBA mice at intervals of one to 10 weeks after inoculation with Giardia muris cysts.

CHAPTER 8

INTRAEPITHELIAL LYMPHOCYTE COUNTS IN CBA
AND CONGENITALLY ATHYMIC (NUDE) MICE
DURING THE COURSE OF PRIMARY INFECTION
WITH GIARDIA MURIS

INTRODUCTION

In the first section of this chapter an experiment is described which was designed to complement the experiments reported in Chapter 7. Thus, intraepithelial lymphocyte (IEL) counts were performed on groups of CBA mice at intervals throughout the course of a primary Giardia muris infection, using an identical protocol to that described in Chapter 7.

In an experiment previously carried out in our laboratory congenitally athymic nude mice (BALB/c nu/nu) were infected with Giardia muris and sacrificed at 1 and 2 weeks after inoculation for studies of villus architecture. IEL counts were performed on uninfected mice and those killed 14 days post-infection. No significant deviation from normal was found in the infected animals but when the experiments described below using CBA mice gave results indicating that the IEL count may rise later than week 3, we made contact with Dr. I. Roberts-Thomson of Melbourne, Australia, who had published work on the inability of nude mice to eliminate giardiasis, in the hope that he might wish to collaborate. This he kindly agreed to do, and he was able to send us histological specimens from mice infected according to an agreed protocol as described below.

EFFECTS OF PRIMARY GIARDIA MURIS INFECTION
ON IEL COUNTS IN CBA MICE (JEJUNUM AND ILEUM)

Groups of 6 CBA mice were infected with 1,000 *Giardia muris* cysts orally as described in Chapter 2. They were sacrificed in groups of 6 at 1, 2, 3, 4, 6, 8 and 10 weeks and specimens of jejunum and ileum were obtained at 10 cm distal to pylorus and 5 cm proximal to ileo-caecal valve respectively, fixed in formol saline and stained with haematoxylin and eosin. Two separate groups of control (uninfected) animals were also studied.

The results are shown in Fig. 8.1. In the jejunum a significant fall in IEL occurred at one week, but by week 3 the counts were significantly raised, and remained so thereafter. In the ileum a significant fall occurred at 3 weeks, and raised counts were obtained from 6 weeks onwards.

EFFECTS OF PRIMARY GIARDIA MURIS INFECTION
ON IEL COUNTS IN CONGENITALLY ATHYMIC NUDE MICE

For this experiment Dr. Roberts-Thomson infected groups of mice with 1,000 *Giardia muris* cysts as follows:

- | | |
|---------|--|
| Group 1 | congenitally athymic nude (BALB/c nu/nu) |
| Group 2 | BALB/c nu/nu reconstituted with 4×10^8 viable lymphoid cells from thymus and mesenteric lymph nodes of BALB/c nu/+ heterozygotes |

Group 3 BALB/c nu/+ (immunologically competent heterozygotes).

Six animals from each group were sacrificed at weeks 1, 3 and 6, and specimens taken from jejunum were fixed in formol saline and stained with haematoxylin and eosin for IEL counts. Three uninfected BALB/c nu/nu mice were killed at weeks 1 and 3 as controls, and specimens taken as described. The IEL counts were read on a coded basis. The results are shown in Fig. 8.2. Uninfected nu/nu mice were found to have IEL counts much lower than nu/+ mice, with reconstituted nu/nu mice showing intermediate values. No change in IEL counts occurred in any of the 3 groups at weeks 1 and 3 after primary infection. At week 6 the count remained low in nu/nu mice, and a modest but statistically insignificant rise occurred in nu/+ mice. The reconstituted nu/nu mice showed a more than twofold increase in IEL between weeks 3 and 6 ($p < 0.01$).

SUMMARY AND CONCLUSIONS

These experiments demonstrated that at the time when trophozoite numbers fell in the jejunum of CBA mice the IEL count rose, and this rise was sustained for at least 10 weeks. In the ileum the IEL count rose later, and again was sustained. The fall in IEL counts at week 1 in jejunum and week 3 in ileum was an unexpected finding. IEL counts in nu/nu mice remained low throughout the course

of the infection, whereas reconstituted nu/nu mice showed a highly significant increase in IEL counts at 6 weeks. No significant change in IEL count was observed in nu/+ mice.

The intraepithelial lymphocyte population has been shown to be composed mainly of T cells, and of these the majority are of the subset associated with suppressor and cytotoxic functions (Selby et al., 1981, Lyscom and Brueton 1982). The increase in numbers of IE lymphocytes at the time of diminution in trophozoite numbers may therefore be due to a population of effector T cells whose function is to attack the parasite either at the epithelial surface or in the intestinal lumen. Owen et al., (1979) showed using scanning electron microscopy that intraluminal trophozoites frequently appear to have lymphocytes attached to their dorsal membranes. In their studies using nude mice Roberts-Thomson and Mitchell (1978) demonstrated that these animals, which are congenitally T cell deficient, did not eliminate giardiasis unless 'reconstituted' with lymphoid cells from an immunocompetent donor of the same strain. There is therefore convincing evidence that the ability to eliminate the parasite is T cell dependent, and that the process may be directly mediated by effector T cells within the intestinal lumen.

GIARDIA MURIS INFECTION OF CBA MICE
IE LYMPHOCYTE COUNTS

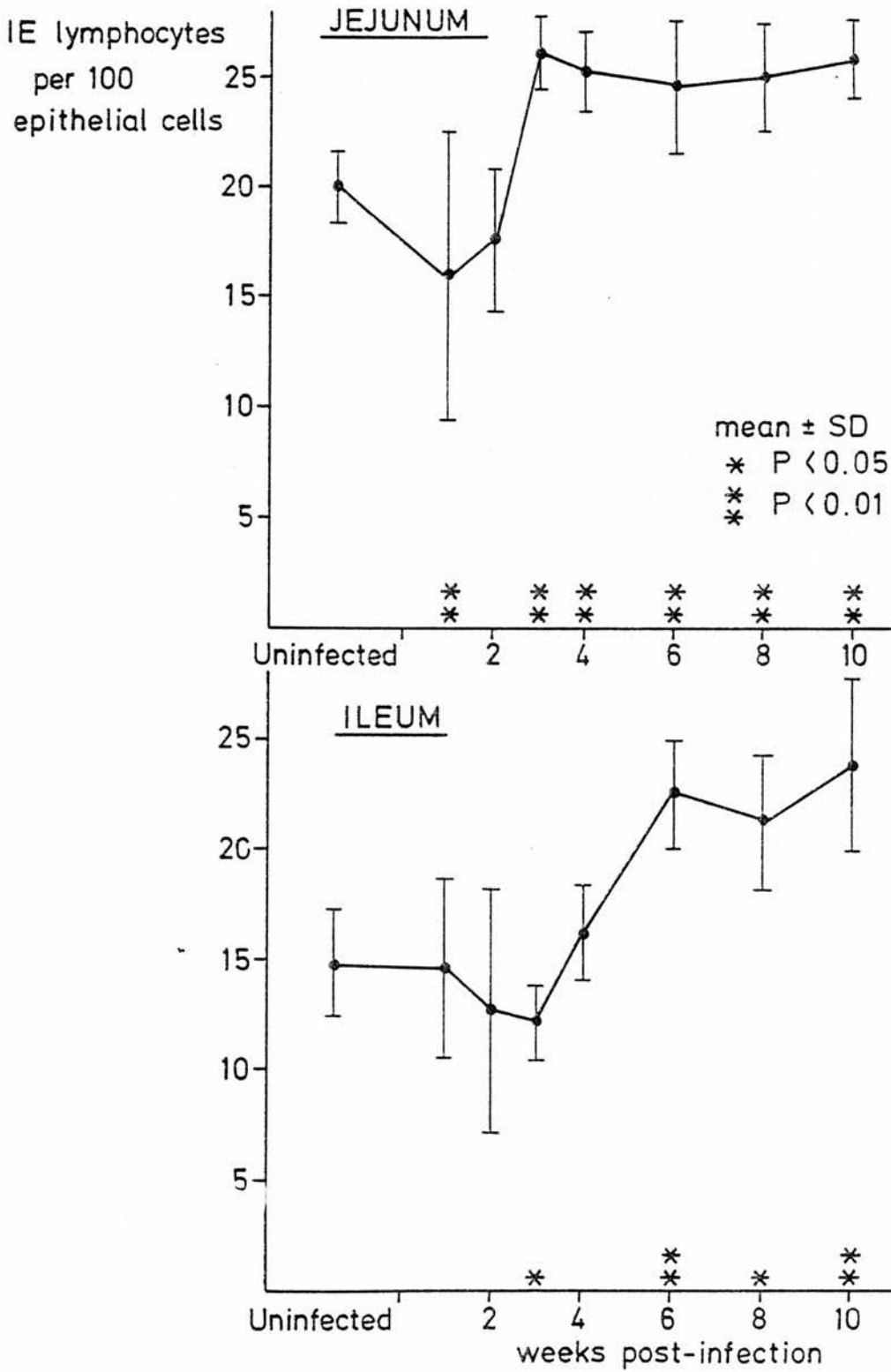


Figure 8.1

Effects of primary *Giardia muris* infection of CBA mice on intraepithelial lymphocyte counts in jejunum and ileum. Six animals per group.

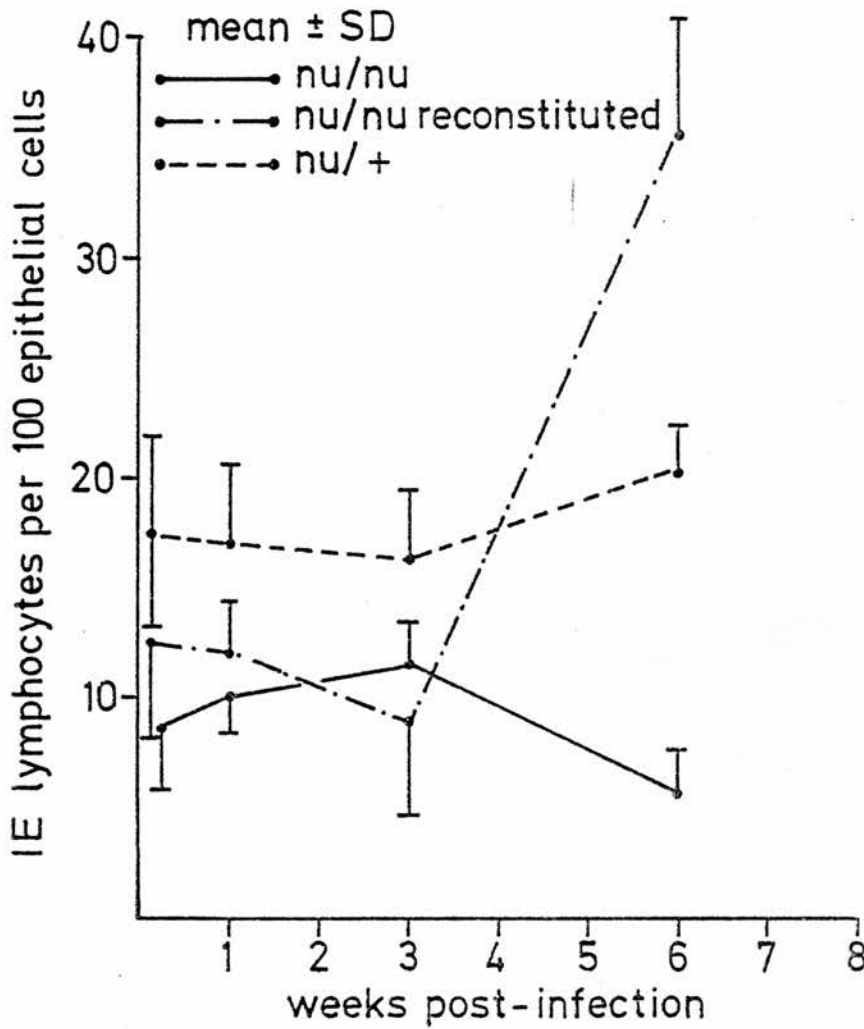


Figure 8.2

Effects of primary Giardia muris infection on intraepithelial lymphocyte counts in jejunum of BALB/c nu/nu mice, BALB/c nu/+ mice, and BALB/c nu/nu mice reconstituted with viable lymphoid cells. Six animals per group.

CHAPTER 9

SERUM ANTIBODIES IN MURINE GIARDIASIS

INTRODUCTION

The constant stumbling-block to serological tests for giardiasis has been the lack of suitable antigenic material. Previous studies in humans relied on crude preparations of cysts or trophozoites (Ridley and Ridley 1976, Vinayak et al., 1978). More recently some success has been obtained in culturing the organism (Meyer 1976; Gillin and Diamond 1980; Visvesvara et al., 1980), but reports of serological studies remain few in number. Attempts to culture *Giardia lamblia* in our laboratory using a modified Diamond's medium and trophozoites obtained from Dr. Marion Ridley (London School of Hygiene and Tropical Medicine), failed repeatedly, and while working in France I therefore set out to develop an immunofluorescence method for the detection of circulating antibodies in mice using as antigen trophozoites taken from the intestine of mice infected only a few days earlier. By using the vibration method for obtaining trophozoites I hoped both by sacrificing the animals early in the course of infection and by using the shortest possible vibration time compatible with obtaining adequate numbers of organisms to obtain a preparation of whole trophozoites free from secretory antibody and intestinal debris. This method provided an entirely satisfactory and predictable source of antigen.

EXPERIMENTAL METHODS

BALB/c mice in batches of 6 were infected with 1,000 *G. muris* cysts orally at the age of 2 - 3 months. The presence of infection was confirmed by performing faecal cyst counts at days 7, 14, 21 and 28. Animals were sacrificed by exsanguination under ether anaesthesia followed by cervical dislocation at days 7, 14, 28, 49, 63 and 98. Trophozoite counts were performed on each animal and serum was stored at -20°C until being tested as described in Chapter 2 for the presence of antibodies to *Giardia muris* trophozoites. Control sera for the immunofluorescence studies were obtained from uninfected BALB/c mice of the same age.

Two additional groups of 5 mice each were studied in order to assess the effects of a second infection on serum antibody levels. Both groups received a primary infection as described above, and then received metronidazole in a dose of 366 mg/Kg/day for 11 days which is known to result in complete cure of murine giardiasis (Mandoul et al., 1961), from day 28 to day 39. Group 1 then received a second inoculation of 1,000 cysts orally on day 56, and were sacrificed 14 days later. Group 2 received a second inoculation on day 93 and were sacrificed 7 days later.

RESULTS

Antibodies of the IgM class were very seldom found during preliminary studies, and when present were at very low titres, so much so that from the preliminary studies onward no attempt was made to detect IgM antibodies.

The method described proved unsatisfactory for the measurement of IgA antibodies, as the trophozoites were often found to be mildly fluorescent even when only fluorescent anti-IgA was applied in the absence of test or control serum. This was taken to mean that the trophozoites were probably coated with secretory IgA even at this early stage. There has since been confirmation that trophozoites taken from the intestine of chronically infected humans were coated with IgA as demonstrated by direct immunofluorescence (Briaud et al., 1981). As a general rule the fluorescence obtained with test sera did not exceed that of controls, only very occasional apparently higher antibody titres being found. This suggests that circulating anti-trophozoite antibody of the IgA class does not usually occur in mice. However, it is possible that the small amounts of IgA already present on the trophozoites was sufficient to mask other antigenic determinants and render the tests invalid even when serum IgA antibody was present.

After a few unsuccessful preliminary studies to establish the correct dilutions of the reagents required, the method proved satisfactory for measuring IgG antibodies,

and the results obtained are shown in Fig. 9.1 and Table 9.1. At day 7 after inoculation only one of the 18 animals tested was positive, at a titre of 4. At day 14, 4 out of 15 animals were positive, with a mean titre of 1.07. By day 28 the majority of mice tested (14 out of 20) had detectable IgG antibody (mean titre 5.9), and at day 49 all 12 animals tested gave positive results (mean titre 41.5). By day 63 the mean titre had fallen to 21.8, with 4 of the 16 animals giving negative results, and at day 98 a further fall in mean titre had occurred to 7.58, with 7 out of 12 animals giving negative results.

In neither of the 2 additional groups did cysts reappear in the faeces following the second inoculation, and only one *Giardia* trophozoite was seen in the intestine of one of the Group 1 animals when sacrificed. These mice therefore appeared to have complete immunity to reinfection with *Giardia muris*. Group 1 (Killed day 70, 14 days after reinoculation) all had IgG antibodies with a mean titre of 60.8, considerably higher than that of the nearest group of animals following primary infection (day 63 - mean titre 21.8), but this difference was not significant. Group 2 (killed day 100, 7 days after reinoculation) was also 100% positive for IgG antibodies, but the mean titre of 8.2 was not significantly different from that of the animals tested on day 93 following primary infection.

SUMMARY AND CONCLUSIONS

A reliable method was developed for the detection of serum IgG antibodies in mice infected with *G. muris*, using as antigen trophozoites obtained from mice infected for less than 5 days. Using this method, antibody titres in mice receiving a primary *G. muris* infection were charted over a 14 week period. By week 4 most animals had detectable antibody, and peak titres were reached at week 7. Subsequently there was a gradual fall in titre to the end of the study at week 14. Two groups of mice infected for a second time at weeks 8 and 13 respectively following a course of metronidazole at week 4 showed complete immunity to reinfection. However, no significant rise in antibody titre occurred in either group in response to attempted reinfection, suggesting that a 'booster' effect did not occur. This in turn suggests that the antibody response reflects the severity of the initial infection, and while it may confer systemic immunity, plays no part in the protective immunity which occurs at the mucosal level.

TABLE 9.1
SERUM ANTIBODIES (IgG) IN MURINE GIARDIASIS

| <u>DAYS</u> <u>POST-INOCULATION</u> | <u>TOTAL</u> <u>NUMBER</u> | <u>NUMBER WITH</u> <u>DETECTABLE</u> <u>ANTIBODY</u> | <u>MEAN</u> <u>ANTIBODY</u> <u>TITRE</u> |
|--|-------------------------------|--|--|
| 7 | 18 | 1 | 0.22 |
| 14 | 15 | 4 | 1.07 |
| 28 | 20 | 14 | 5.90 |
| 49 | 12 | 12 | 41.50 |
| 63 | 16 | 12 | 21.80 |
| 70* | 5 | 5 | 60.80 |
| 98 | 12 | 5 | 7.58 |
| 100** | 5 | 5 | 8.20 |

* Metronidazole treated, reinoculated 14 days previously

** Metronidazole treated, reinoculated 7 days previously

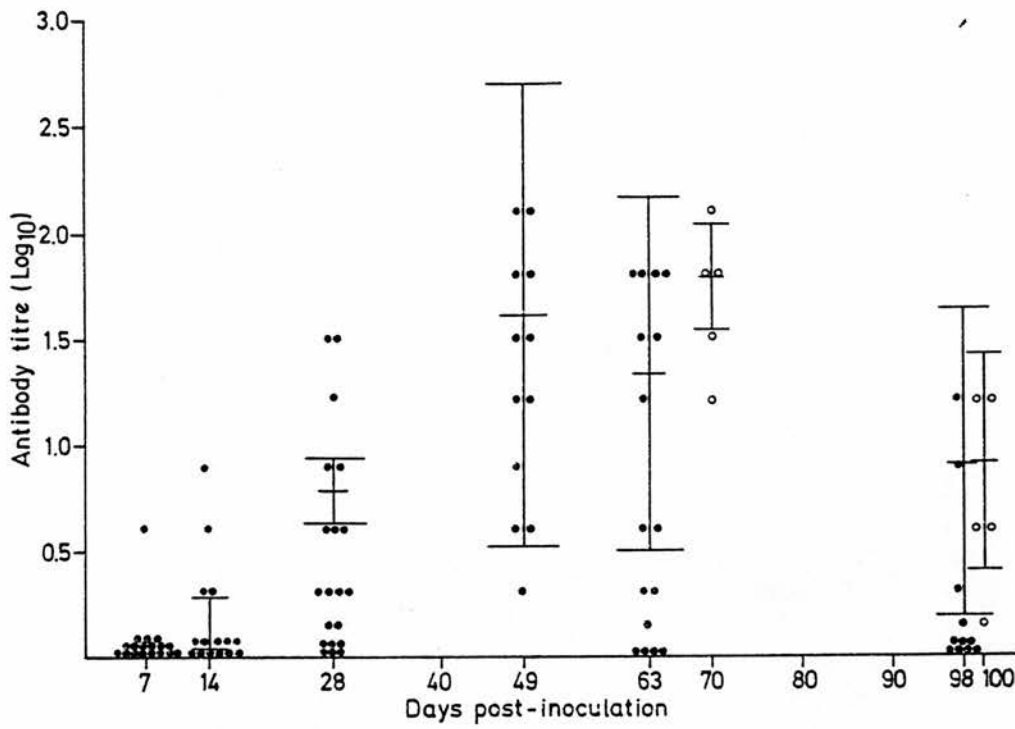


Figure 9.1

Changes in serum antibody titre (IgG) with time following primary (closed circles) and secondary (open circles) infection with Giardia muris (mean \pm SD).

CHAPTER 10

GENERAL DISCUSSION

In this thesis I have examined several aspects of giardiasis in the hope of being able to shed some light on the mechanisms whereby this organism causes disease. I began by cataloguing my own experience of the disease in a gastrointestinal unit in a temperate country. This is an unusual setting from which to study giardiasis since the disease is commonest where sanitation is poor or where groups of individuals (e.g. children in care) are at risk because of poor hygiene. Most studies therefore originate in endemic areas, or from institutions interested in tropical and infectious diseases, and deal mainly with acutely infected individuals. My aim was to see whether adults with giardiasis presenting in this country had features suggesting possible reasons for their developing a chronic or persistent form of the disease, and whether the pathological features differed in any way from those seen in other parts of the world.

These clinical studies showed, firstly, that giardiasis is not common in adult gastroenterological practice in this country. Only 8 such patients presented to this unit in a 2 year period, and of these only 4 appeared to have contracted the disease in this country. This does not necessarily constitute evidence that giardiasis is rare in the community. Rendtorff (1954), studying human volunteers whom he infected with cysts given in capsule form, demonstrated that in normal individuals the infection is usually short-lived (as judged by faecal cyst excretion) and frequently asymptomatic. Recent epidemiological surveys

have estimated the incidence of the disease in temperate countries at between 3 and 7% of the population (Petersen 1972, Quinn 1971). These studies relied on faecal cyst counts for case detection, a method which is notoriously unreliable (Ament and Rubin 1972, Kamath and Murugasu 1974), and almost certainly underestimate the true incidence of the disease very considerably. Using cultured trophozoites as antigen in an enzyme-linked immunoassay Smith et al. (1981) found evidence of specific antibodies, suggesting previous infection, in 14% of an apparently healthy sample of individuals in Washington State. No epidemiological studies have been performed in the U.K., but *Giardia lamblia* was cited as the most commonly reported bowel pathogen in the Communicable Disease Weekly Reports of 1977. During one year of the present study 48 cases were detected by stool microscopy at the Western General Hospital, and for the past 4 years it has been the most frequently detected pathogen in this laboratory (Dr. I. Gould and Dr. K.C. Watson, personal communication).

The patients described in Chapter 4 who developed chronic symptomatic giardiasis in this country therefore almost certainly represent the tip of the iceberg, the majority of patients with acute infection remaining in the community. I found that these patients differed little from those described in previous series in terms of the pathological effects of the infection. Although none presented with a malabsorption syndrome there was evidence of mild malabsorption of fat in the majority, the degree of

abnormality bearing no relationship to the histological appearances of the jejunum. This is in conflict with previous published series in which the degree of malabsorption was found to correlate roughly with the extent of the mucosal damage (Hoskins et al., 1967, Wright et al., 1977), but this discrepancy can be explained by the small numbers in the present study, the generally mild degree of mucosal abnormality, and the inclusion of 2 patients with previous gastric surgery who had moderate fat malabsorption with virtually normal histological appearances. The spectrum of histological abnormality seen in these patients was, however, comparable with previous series, ranging from normal to partial villus atrophy (Yardley et al., 1964, Hoskins et al., 1967, Barbieri et al., 1970, Wright et al., 1977, Duncombe et al., 1978).

Morphological measurements in the jejunal mucosa of these patients failed to show significant differences from controls, or in patients before and after treatment, but this may simply reflect the small numbers studied.

Although it seems likely that more mucosal damage will accrue in those patients with chronic infections, as, for example, in immunodeficient subjects, it is impossible to extract this information from the literature, since the exact duration of infection and its relation to the pathological appearances can only be guessed at. Had Rendtorff (1954) been able to perform jejunal biopsies on his human volunteers this information might now be available,

but such studies would now be considered unacceptable on ethical grounds. Some help in resolving this point has been obtained from the animal studies reported here and elsewhere. The experiments described in Chapter 6 of this thesis can be considered analogous to those of Rendtorff, since normal hosts were infected acutely with a known number of cysts. This is therefore a model of acute infection in the previously normal individual.

The changes in intestinal architecture in mice early in the course of the infection consisted mainly of a very limited increase in crypt mitosis with villi of normal length. This is similar to the pattern of intestinal architecture which has been observed in chronic murine giardiasis (MacDonald and Ferguson 1978), and is also similar to chronic graft-versus-host disease in mice (Mowat and Ferguson 1981). Mucosal changes in graft-versus-host disease and in allograft rejection are caused by cell-mediated hypersensitivity (MacDonald and Ferguson 1977, Mowat and Ferguson 1981). These authors proposed that the effects on crypt cell mitosis may be caused by 'enteropathic' lymphokines secreted by T lymphocytes, and that a more vigorous cell-mediated reaction may lead to villus damage. Roberts-Thomson and Mitchell (1978) provided evidence that T cell-mediated mucosal injury similarly occurs in giardiasis by demonstrating that the changes in the villus-crypt ratio of giardia infected athymic (nude) mice were augmented when the mice were reconstituted with lymphoid cells from uninfected donor mice.

The changes in small bowel architecture late after infection (weeks 8 and 10), consisted, in the jejunum, of an expansion in villus size with increased disaccharidase activity, and in the ileum of a diminution in villi and crypts with reduced disaccharidases. An acute insult to the jejunal mucosa therefore appears to result in a rebound increase in mucosal volume and digestive capacity, but the present work provides no clue to the mechanism underlying this trophic effect. The changes in the ileum were the inverse of those in the jejunum, and demonstrate the remarkable adaptive properties of the ileum.

Vitamin B₁₂ malabsorption was a common finding in the patients studied, appearing more consistently than in previous reports (Antia et al., 1966, Ament and Rubin 1972, Wright et al., 1977, Tandon et al., 1977), although anaemia due to B₁₂ deficiency did not occur. Again there was no correlation with the degree of mucosal abnormality in the jejunum, and elimination of the parasite promptly restored B₁₂ absorption to normal. This finding is at variance with previous reports, in which vitamin B₁₂ malabsorption has been associated almost exclusively with those patients showing significant villus atrophy (Wright et al., 1977, Ament and Rubin 1972). A high proportion (but not all) of these patients have been shown to have concomitant bacterial colonisation of the small intestine (Tomkins et al., 1978, Tandon et al., 1977), and it has been suggested that the vitamin B₁₂ malabsorption can be ascribed either to the bacterial overgrowth or to the degree

of mucosal damage. Wright et al., (1977) were, however, unable to show any significant improvement in B₁₂ absorption in their patients treated initially with tetracycline, whereas metronidazole and mepacrine were found to be equally efficacious in this respect.

Most of my patients had B₁₂ malabsorption in the absence of severe mucosal abnormality, and the response to treatment, where tested, was complete. Taken with the data presented in Chapters 6 and 7 of this thesis, demonstrating that the organism is seldom found in great numbers in the ileum (the site of vitamin B₁₂ absorption) and that this area is free of the pathological changes noted in the jejunum at the height of the infection, these observations suggest that the parasite itself may utilise the vitamin in quantities sufficient to render the Schilling test abnormal. This is consistent with Scudamore's observation (1961) that Giardia trophozoites appear to require vitamin B₁₂ for growth.

Disaccharidase deficiency was common in my patients, and in this case the severity was in general comparable with the degree of histological abnormality observed. As had been noted by previous authors (Hoskins et al., 1967, Cain et al., 1968, Jennings et al., 1976, Duncombe et al., 1978) disaccharidase deficiencies - particularly hypolactasia - may occur in the presence of minimal histological abnormality. Such observations in humans provide little insight into the mechanism producing this abnormality, a question to which I addressed myself when working with the animal model of the disease. By measuring disaccharidase values in both

jejunum and ileum at intervals throughout the course of a primary infection, I found that disaccharidase levels were lowest in the jejunum at the time when trophozoite numbers were highest, even though at this time there was little abnormality of villus architecture or epithelial cell kinetics. In the ileum, where few parasites were found, the disaccharidase levels rose at this time, suggesting rapid ileal adaptation in the presence of disaccharide malabsorption in the jejunum. The abnormalities in the jejunum returned to normal as parasite numbers declined, and later the disaccharidase content of the mucosa became supra-normal as the villus length increased. It therefore seems likely that the disaccharidase deficiency of giardiasis is due to the direct effect of the trophozoites on the brush border of the enterocytes. Whether this is due to a simple mechanical effect or to a substance elaborated by the organism is not known, but experimental work using artificially created blind loops has shown that some species of bacteria contain, and are thought to secrete, substances which cause disaccharidase deficiency (Jonas et al., 1978). The substances implicated in this toxic reaction are thought to be proteases, and these authors suggested that other intestinal pathogens known to be associated with disaccharidase deficiency might act in the same way. My findings support this view, which could be tested by means of a suitably adapted animal model.

It has been shown that patients with hypogammaglobulinaemia tend to develop more severe villus damage and consequent

malabsorption when infected with Giardia (Ament and Rubin 1972) and it may therefore be reasonable to suppose that the small proportion of giardiasis patients with apparently normal immunity who develop chronic infection, with or without villus damage and malabsorption, may have some underlying defect in mucosal immunity.

On the basis of studies using jejunal secretions from infected humans, Zinneman and Kaplan (1972) suggested that these patients may have a pre-existing deficiency of secretory IgA. This stimulated several studies of the immunoglobulin-containing cell population of the jejunal mucosa, with conflicting results. Popovic et al., (1974) also found decreased IgA in jejunal juice both before and after treatment, and in addition recorded depressed numbers of IgA containing cells at the time of the infection. Totally conflicting results were reported by Thomson et al., (1977), Blenkinsopp et al., (1978) and Ridley and Ridley (1976). The results of the Ig-containing cell counts described in Chapter 5 are of particular interest since, for the first time, all 5 immunoglobulin classes were studied. There was a tendency towards a reduction in IgA cells at the time of infection, which, although not statistically significant, does lend support to the earlier studies. Patients had higher numbers of IgM cells than controls, and although this did not reach statistical significance it is of interest in the light of Ridley and Ridley's observation that this may indicate an early stage of the infection. Giardiasis is

not uncommon in Lyon, and it seems quite likely that these patients would mainly be suffering from acute infections (the only information available to me was that none had malabsorption). I was later able to study conventionally fixed and stained jejunal biopsy specimens from these patients, and found no evidence of villus atrophy, though I did not perform formal morphological measurements. Only one had a slightly raised IE lymphocyte count, and this would also tend to indicate that most had mild disease.

The most striking finding in this study of the Ig-containing cells in the jejunal mucosa was the highly significant increase in IgE-containing cells (many of which may have been mast cells) at the time of infection. Mast cells have been shown to play an important part in cell-mediated hypersensitivity reactions involving T lymphocytes (Askenaze et al., 1980). T cells are thought to be of central importance in the normal host response to Giardia infection, as discussed below, and the finding of a local IgE or mast cell response demonstrates another facet of the integrated response to the parasite. In certain circumstances it seems that this local IgE stimulation can become systematised, for reports exist of various allergic phenomena occurring in association with giardiasis (Fossati 1971, Lopez-Brea et al., 1979, Harris and Mitchell 1949, Carroll et al., 1961, Dellamonica et al., 1976, Goobar 1977, Weisman 1979), and one patient in Lyon had bronchial asthma which resolved with treatment of his giardiasis. It is not known whether these individuals are typically atopic.

One further aspect of this immunofluorescence study deserves comment, and that is that after treatment of the infection not only did the IgE cell numbers return to normal, but there was also a significant increase in IgA cell numbers at 1 month post-treatment. This suggests the possibility that the parasite may in some way suppress local production of IgA, and might explain the findings of Zinneman and Kaplan (1972) and Popovic et al., (1974). It is discussed further in relation to changes in IE lymphocyte counts (below).

Intra-epithelial lymphocyte counts, in contrast to the absorption studies, were only raised in those patients with a degree of partial villus atrophy. Wright and Tomkins (1977), however, showed that the IE lymphocyte count usually correlates with the degree of malabsorption in patients with giardiasis, and they went on to demonstrate that the counts return to normal on successful treatment of the infection. In my own series the initially raised IE lymphocyte counts also returned towards normal in parallel with the histological appearances within one month of successful treatment.

Wright and Tomkins (1977) suggested that the mucosal damage which they observed in giardiasis may be due to a hypersensitivity state analogous to coeliac disease, citing as evidence the similar numbers of IE lymphocytes in relation to the apparent mucosal abnormality. This notion is based on the evidence that the villus atrophy of coeliac disease is largely caused by cell-mediated hypersensitivity, and that the increase in numbers of IE lymphocytes is central to

this abnormal immune reaction (Ferguson and Jarrett 1975, MacDonald and Ferguson 1977).

The studies of sequential IE lymphocyte counts in mice infected with Giardia muris demonstrated that the rise in IE lymphocyte numbers coincides with the decline in the parasite population. This may be taken as further evidence for the importance of T cells in the host immune response to Giardia, a view further strengthened by the findings when similar studies were performed using athymic nude mice, heterozygous nude mice, and homozygous nude mice reconstituted with viable lymphocytes from syngeneic donors. This rise in IE lymphocyte numbers was found to be sustained for the duration of the study (10 weeks), suggesting that these cells play an important part in host resistance to reinfection.

An unforeseen finding was the significant fall in IE lymphocyte numbers early in the course of the infection. Since this is approximately contemporaneous with the fall in brush border disaccharidases it may represent a direct 'toxic' effect of the parasite. The immunofluorescence studies in humans have also indicated a possible reduction in mucosal IgA synthesis at the time of the infection, and the parasite may therefore have the ability to suppress host immunity at least in the short term. This is highly speculative and would require further study for confirmation.

Recent studies have shown that IE lymphocytes are different from peripheral blood T lymphocytes in respect of their surface markers, and therefore presumably also

their functional characteristics (Selby et al., 1981, Lyscom and Brueton 1982). About 70% of these cells possess surface markers associated with the suppressor-cytotoxic phenotype, and it is therefore possible that these cells are cytotoxic T cells which cross the epithelium in order to attack the parasite within the intestinal lumen. This is supported by the scanning electron microscope studies of Owen et al., (1979), in which they demonstrated small lymphocytes adhering to Giardia trophozoites in the lumen. The intestinal damage associated with giardiasis could therefore be due to lymphokines released during this cytotoxic reaction, and this fits with the observation that the IE lymphocyte count is highest in those patients with the most severe mucosal damage. Whether this is in any way analogous to the pathogenesis of coeliac disease is a matter for speculation.

Much of what has been said suggests the possibility that patients with giardiasis may fall into two broad groups: those who, like the normal, healthy volunteers in Rendtorff's study (1954), have a short-lived infection causing few or no symptoms and conferring immunity, and

those who do not rid themselves of the infection, developing variable degrees of intestinal damage. Since there is no information on genetic markers such as HLA antigens in giardiasis patients, it is speculative to suggest that they may be genetically predisposed. However, Roberts-Thomson and Mitchell (1978) have shown that different strains of mice eliminate the infection at different rates, providing very strong evidence that the efficacy of the immune response to the organism is genetically determined. At its most extreme, the congenitally athymic nude mice were shown not to be able to eliminate Giardia muris at all unless transfused with immunocompetent cells from heterozygous animals (Stevens et al., 1978). There are no reports of giardiasis in the comparable human syndrome of thymic aplasia (Di George's syndrome), a condition which presents in early childhood and is associated with a very poor prognosis.

The mice used in the studies in this thesis were of the strains CBA and BALB/c, with the exception of the nude mice in which IE lymphocyte counts were performed (Chapter 8). Both of these groups of mice were shown by Roberts-Thomson and Mitchell (1978) to eliminate a primary infection relatively rapidly, and this was confirmed. Although the more sophisticated methods developed for this thesis have shown that up to 50% of these animals may harbour very small numbers of vegetative organisms for long periods of time thereafter, it is probably reasonable to assume that the primary infection in these mice, in which the infection

may occur naturally, is analogous to acute infection in immunocompetent, healthy humans.

The sequential studies in these mice indicated that the few parasites remaining after apparent self-cure are not important in conferring immunity to reinfection. Although they may evade the host's immune system for long periods of time, their numbers appear to be held in check by mucosal immune mechanisms, as was shown by the magnitude and rapidity of the recrudescence of infection which occurred when these mice received parenteral corticosteroids.

The theory that chronic infection develops as a result of subtle defects in host immunity is attractive, and its case is strengthened by the demonstration that in a primary infection in mice, parasite numbers can be increased at all stages, and chronicity induced, by immunosuppression with corticosteroids (Chapter 6). However, other factors may affect the individual's ability to rid himself of the parasite, and in the present series 2 of the patients had previously undergone gastric surgery for peptic ulceration. This association has been reported previously (Vachon et al., 1963, Yardley et al., 1964, Gianella et al., 1973), but the suggestion that it is due to the reduction in gastric acidity seems facile, since gastric acid may be necessary for excystation (Bingham and Meyer 1979), and is in any case almost certainly not relevant to the host's attempts to eliminate the parasite once it has become established in the upper small intestine. Although the organism can gain access to, and cause disease

of, the biliary tract and pancreas (Goldstein et al., 1978, Koszarska et al., 1977), it is not known whether chronicity may be induced by colonies of trophozoites sequestered in these extra-intestinal sites. It is perhaps surprising that there have been no reports of giardiasis in intestinal blind loops, for example in patients who have had jejunio-ileal bypass for morbid obesity. This suggests that physical factors within the intestine are probably of little importance in determining the rate of elimination of the parasite, and the question of why post-gastrectomy patients may be prone to chronic giardiasis remains open.

The evidence that the magnitude of a primary infection and rate of its eradication are determined mainly by the immune response of the host is now overwhelmingly convincing. While there is no doubt that T lymphocytes are of central importance to the mucosal immune defence mechanisms, however, the demonstration of a systemic antibody response (Chapter 9) is more difficult to assess in terms of its significance.

It is known that humans with chronic giardiasis causing malabsorption produce a systemic IgG antibody to cysts. Ridley and Ridley (1976) used a crude preparation of G. lamblia cysts, taken from patients' faeces, as antigen in an immunofluorescence test. They found IgG antibodies in 34/36 cases with malabsorption, but 0/2 cases without malabsorption. There was some correlation between antibody titre and the degree of histological abnormality

in the jejunum. The test was also positive in 10/34 patients in whom no Giardia cysts or trophozoites could be detected. Similar results were reported by Vinayak et al., (1978), using sonicated human-derived cysts as antigen in a gel immunodiffusion test, but fewer cases were studied. They provided no details of the presence or absence of malabsorption, nor of jejunal histology.

These crude cyst antigens were generally considered unsatisfactory, and more recent studies have used cultured trophozoites as antigen. By using these trophozoites either in an immunofluorescence test (Visvesvara et al., 1980) or in an enzyme-linked immunosorbent assay (Smith et al., 1981) IgG antibody has been shown to occur in the majority of humans with symptomatic giardiasis. Although Visvesvara et al., found some cross-reaction with patients having other parasite infections the titres found in these patients did not overlap with the giardiasis patients, the majority of whom had titres of 64 to 128, and absorption studies confirmed that the antibody was specific for Giardia. They suggested that the low titres found in these other patients may have been the result of previous infection. Smith et al., also showed that most symptomatic patients have antibody titres of around 64 (81% of 59 sera studied), but a few of their patients had titres as high as 2048. They were able to study paired acute and convalescent sera in 15 patients. Antibody titre was invariably higher in the acute sample, but 11 of these patients still had detectable antibody between 2 weeks and 15 months later.

There was seldom any fall in titre during the first 3 to 4 months, but those patients tested later after treatment of the infection tended to have lower antibody titres. Again the specificity of these antibodies for Giardia lamblia was confirmed by absorption studies. By comparing the results with immunofluorescence tests performed on the same sera they demonstrated that the enzyme-linked method was more sensitive and gave higher titres.

The significance of these antibodies in relation to host immunity to subsequent infection is not known. The results of the present study, however, and the observation of Smith et al., (1981) that patients with high antibody titres could have recurrent infections, suggest that the systemic antibody response may be an epiphenomenon of little biological significance. Nevertheless, serum antibody estimations may yet prove to be useful both in diagnosis of active infection and in epidemiological surveys. Further work is necessary to establish the pattern of antibody response with time following initial exposure, though the present data suggest that the circulating antibodies may persist for a relatively long period of time.

While they may play no part in terminating the primary infection these antibodies could however still be important in protecting against subsequent infections. Roberts-Thomson and Mitchell (1979) showed that systemic immunisation of mice with Giardia trophozoites in complete Freund's adjuvant protected against infection when the mice

were subsequently challenged orally with viable cysts. Nevertheless it is impossible to be certain that this protective immunity was a function of the induced systemic antibody response, as opposed to a cellular immune response which is more difficult to quantify. Similarly, studies showing that new-born mice are protected from giardiasis when suckling the milk of their previously infected mothers (Stevens and Frank 1978, Andrews and Hewlett 1981) have failed to establish that the protection is due solely to the IgA and IgG antibodies which the authors have demonstrated, and not to the simultaneous transfer of lymphocytes. A recent report that systemic IgG antibodies have opsonising effects on the ingestion of Giardia trophozoites by macrophages in an in vitro test does, however, suggest that the immune response to the parasite involves both cellular and humoral limbs (Radulescu and Meyer 1981).

The data presented in this thesis demonstrate the multi-faceted nature of the immune response to Giardia organisms. The importance of T lymphocytes, as evidenced by the numbers of IE lymphocytes, has been confirmed and the dynamics of that part of the response described. IgE containing cells were shown to be present in the intestinal mucosa in raised numbers at the time of infection, and this can be taken as further evidence for a local cell-mediated immune response rather than an immediate hypersensitivity state. The development of a systemic IgG antibody response to the parasite has been documented, though its significance in terms of host protection remains uncertain; nevertheless,

new methods for detecting these specific antibodies will greatly facilitate epidemiological surveys of this world-wide intestinal infection.

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